THE BIOSYNTHESIS OF ADRENAL C11-OXY C_{21} STEROIDS, IMPLICATED IN 21-HYDROXYLASE DEFICIENCY – 21-DESOXYCORTISOL AND 21-DESOXYCORTISONE AND THEIR DOWNSTREAM METABOLISM

by
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March 2017

ABSTRACT

Adrenal C_{19} steroids are often implicated in numerous androgen dependent disease conditions. Androgen excess is the hallmark of 21-hydroxylase deficiency (21OHD) fuelled by an increase in the production of adrenal androgens and androgen precursors. In addition, increased levels of progesterone (P4) and 17α-hydroxyprogesterone (17OHP4), the substrates of the defective cytochrome P450 steroid 21-hydroxylase, have also been reported. Adrenal steroids are secreted into circulation, for further downstream metabolism. Conversion of adrenal steroids to active androgens in peripheral target tissue is dependent on the tissue specific expression of key enzymes, which significantly influence steroid profiles at cellular level, ultimately influencing homeostasis in androgen responsive tissue. In 21OHD, the conversion of P4 and 17OHP4 in an alternative metabolic pathway to the potent androgen, dihydrotestosterone, is reported, which has been described as the backdoor pathway.

The accumulation of P4 and 17OHP4 furthermore present substrates for 11β-hydroxylation by the cytochrome P450 11β-hydroxylase (CYP11B) isozymes. In this study the catalytic activity of the CYP11B isozymes towards P4 and 17OHP4 are presented. CYP11B1 and CYP11B2, also termed cytochrome P450 aldosterone synthase, transiently expressed in HEK293 cells, effectively utilised P4 and 17OHP4 as substrates, yielding 11β-hydroxyprogesterone (11OHP4) and 21-desoxycortisol (DOF). Catalytic conversions of DOF and 21-desoxycortisone (DOE), the C11-keto derivative of DOF, together with P4 and 17OHP4, were assayed in HEK293 cells transiently transfected with steroid 5α-reductase type 1 or type 2 (SRD5A1 or SRD5A2). Conversion of DOF, DOE, P4 and 17OHP4 by the SRD5A isozymes, yielded 5α-pregnan-11β, 17α-diol-3, 20-dione (11OHPdione) and 5α-pregnan-17α-ol-3, 11, 20-trione (11KPdione), dihydroprogesterone and 5α-pregnan-17α-ol-3, 20-dione (Pdione), respectively. We identified these novel steroids, 11OHPdione and 11KPdione, which were converted to the novel products 5α-pregnan-3α, 11β, 17α-triol-20-one and 5α-pregnane-3α, 17α-diol-11, 20-dione by 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2) catalysed conversions in transiently transfected HEK293 cells by accurate mass determination. Metabolism of DOF in LNCaP cells, yielded DOE, 11β-hydroxyandrosterone and 11-ketoandrosterone, indicative of the conversion by endogenous 11β-hydroxysteroid dehydrogenase type 2, SRD5A, AKR1C2 and cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17A1) enzymes. These findings shows that DOF, produced in 21OHD, can be metabolised via the C11-oxy Pdione en Pdiol (5α-pregnan-3α, 17α-diol-20-one) intermediates to suitable substrates for the lyase activity of CYP17A1 thus leading to the production of C11-oxy C_{19} steroids.

Taken together, the biosynthesis of C11-oxy C_{21} steroids, together with their metabolism by the enzymes in the backdoor pathway, yielded novel steroid metabolites contributing to the pool of potent androgens in 21OHD, with said steroids also presenting possible biomarkers in disease identification.

ABSTRAK

Adrenale steroïede word dikwels geïdentifiseer as belangrike rolspelers in verskeie androgeen-afhanklike siektetoestande. Androgeen oormaat word deur die bank tydens 21-hidroksilase gebrek (21OHD) waargeneem, met stygings in die bloedvlakke van adrenale androgene en androgeenvoorlopers. Daarbenewens, word verhoogde vlakke van sitochroom P450 steroïed 21-hydroksilase (CYP21A2) substrate, progesteroon (P4) en 17α-hidroksiprogestoroon (17OHP4), ook waargeneem. Adrenale steroïede word in sirkulasie vrygestel en word verder gemetaboliseer. Die produksie van aktiewe androgene vanaf adrenale steroïede in teiken weefsel is afhanklik van die uitdrukking van sleutel ensieme in daardie weefsel wat die androgeenprofiel op sellulêre vlak sal beïnvloed en uiteindelik sal bydra tot homeostase in weefsel wat androgeen sensitief is. Die metabolisme van P4 en 17OHP4 na die aktiewe androgeen, dihidrotestosteroon, in ’n alternatiewe metaboliese padweg, is reeds beskryf in 21OHD, genaamd die ‘agterdeur’ padweg.

In die bynier dien P4 en 17OHP4 in 21OHD as substrate vir 11β-hidroksilasie deur die sitochroom P450 11β-hidroksilase (CYP11B) isosieme. In hierdie studie is die katalitiese aktiwiteit van die CYP11B isosieme teenoor P4 en 17OHP4 ondersoek. Beide isosieme CYP11B1 en CYP11B2, oftewel sitochroom P450 aldosteroon sintase, uitgedruk in HEK293 selle, het P4 en 17OHP4 effektief gekataliseer en onderskeidelik na 11β-hidroksiprogesteroon (11OHP4) en 21-desoksikortisol (DOF) omgesit. Die katalitiese omskakelings van DOF en 21-desoksikortisoon (DOE), die C11-keto derivaat van DOF, tesame met P4 en 17OHP4 deur steroïed 5α-reduktase tipe 1 en tipe 2 (SRD5A1 en SRD5A2) uitgedruk in HEK293 selle is uitgevoer. In die SRD5A gekataliseerde reaksies is DOF, DOE, P4 en 17OHP4 onderskeidelik omgeskakel na 5α-pregnan-11β, 17α-diool-3, 20-dioon (11OHPdioon) en 5α-pregnan-17α-ol-3, 11, 20-trioon (11KPdioon), dihidroprogesteroon en 5α-pregnan-17-ol-3, 20-dioon (Pdioon). Beide hierdie steroïede, 11OHPdioon en 11KPdioon, tesame met 5α-pregnan-3α, 11β, 17α-triool-20-oon en 5α-pregnane-3α, 17α-diool-11, 20-dioon, die onderskeidelike produkte van die 3α-hidroksisteroïed dehydrogenase tipe 3 (AKR1C2) gekataliseerde reaksies, ook uitgedruk in HEK293 selle is geïdentifiseer met behulp van akurate massa bepalings. Hierdie vier steoïede is tot op datum nog nie voorheen beskryf nie. Die metabolisme van DOF in LNCaP selle het DOE, 11β-hidroksiandrosteroon en 11-ketoandrosteroon gelewer, reaksies gekataliseer deur endogene 11β-hidroksisteroïed dihidrogenase tipe 2, SRD5A, AKR1C2 en sitochroom P450 17α-hidroksilase/ 17, 20 lyase (CYP17A1) ensieme. Hierdie bevinding dui daarop dat DOF, in 21OHD gemetaboliseer word via die C11-oksi Pdioon en Pdiool (5α-pregnan-3α, 17α-diool-20-oon) intermediate, geskikte substrate is vir die liaseaktiwiteit van CYP17A1 en waarvan die eindprodukte die C11-oksi C_{19} steroïede is.

Ten slotte, die biosintese van C11-oksi C_{21} steroïede, tesame met hul metabolisme deur die ensieme in die ‘agterdeur’ padweg, lewer unieke steroïede wat bydrae tot aktiewe androgeenvlakke in 21OHD, steroïede wat ook as potensiële biomerkers vir die siekte dien.

DEDICATION

"Make a careful exploration of who you are and the work you have been given, and then sink yourself into that. Don't be impressed with yourself. Don't compare yourself with others. Each of you must take responsibility for doing the creative best you can with your own life. Be very sure now, you who have been trained to a self-sufficient maturity, that you enter into a generous common life with those who have trained you, sharing all the good things you have and experience."

Galatians 6: 4-6, The Message

Dedicated to a God that never stops romancing my heart. You have blessed me beyond measure, may Your work be evident in everything I do.

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TABLE OF CONTENTS

DECLARATION ii
ABSTRACT iii
ABSTRAK iv
DEDICATION v
ACKNOWLEDGEMENTS vi
TABLE OF CONTENTS vii
LIST OF FIGURES iix
LIST OF TABLES xi
ABBREVIATIONS xii
General xii
Enzymes xiii
Steroid hormones xiv

CHAPTER 1 INTRODUCTION 1

CHAPTER 2 ADRENAL STEROIDOGENSIS 4

2.1 Introduction 4
2.2 Overview of the adrenal gland 4
2.3 Regulation of adrenal steroid production 6
2.4 Biosynthesis of adrenal steroid hormones from cholesterol 7
2.5 Enzymes catalysing the biosynthesis of adrenal steroid hormones 8
2.5.1. The cytochrome P450 enzymes 10
2.5.2 The hydroxysteroid dehydrogenase enzymes 12
2.6 Adrenal steroidogenesis in 21OHD 14
2.6.1 Adrenal androgen steroid production during 21OHD 15
2.7 Screening of 21OHD 16
2.8 Treatment strategies 18
2.9 Summary 18

CHAPTER 3 DOWNSTREAM PATHWAYS IN THE METABOLISM OF ADRENAL ANDROGEN PRECURSORS 19

3.1 Introduction 19
3.2 Pathways involved in androgen metabolism 19
3.2.1 The conventional C_{19} pathway 20
3.2.2 The alternative 5α-dione pathway 21
3.2.3 The 11OHA4 pathway 22
3.2.4 The backdoor pathway 24
3.3 Androgen inactivation 27
3.3.1 AKR1C2 27
3.3.2 UGT2B enzymes 28
3.4 Summary 29

CHAPTER 4 THE BIOSYNTHESIS OF C11-OXY C_{21} STEROIDS BY ADRENAL CYP11B1 AND CYP11B2 ENZYMES 30

4.1 Methodology 32
4.1.1 Materials 32
4.1.1.1 Cell lines and vectors 32
4.1.2 Extraction of plasmid DNA 32
4.1.3 Enzyme assays in transiently transfected HEK293 cells 33
4.1.4 LC-MS/MS analyses of steroids 33
4.1.4.1 Accurate mass determination of 11β-hydroxylated steroids 33
4.1.4.2 Separation and quantification of steroid metabolites using UHPLC-MS/MS 34
4.1.5 Statistical analyses 35
4.2 Results 35
4.2.1 Plasmid DNA analyses 35
4.2.2 LC-MS/MS steroid analysis of the C11-oxy C_{21} steroids 35
4.2.2.1 Accurate mass-determination of 11OHP4 and DOF 35
4.2.2.2 UHPLC-MS/MS analyses of steroid metabolites 37
4.2.3 Biosynthesis of 11OHP4 and DOF in HEK293 cells expressing CYP11B 39
4.3 Discussion 43

CHAPTER 5 THE METABOLISM OF DOF AND DOE 47

5.1 Methodology 49
5.1.1. Materials 49
5.1.1.1 Cell lines and vectors 49
5.1.2 Purification of plasmid DNA 50
5.1.3 Enzyme assays in transiently transfected HEK293 cells 50
5.1.4 DOF and DOE metabolism in LNCaP, PNT2 and PC3 cells 51
5.1.4.1 Deconjugation 51
5.1.5 Steroid extraction 51
5.1.6 LC-MS/MS analyses of steroid metabolites 51
5.1.6.1 Accurate mass determination of novel C11-oxy C_{21} steroids 51
5.1.6.2 UHPLC-MS/MS separation and quantification of steroid metabolites 52
5.1.6.3 Separation and quantification of steroid metabolites using UPC²-MS/MS 53
5.1.7 Statistical analyses 54
5.2 Results 54
5.2.1 Plasmid cDNA analyses 54
5.2.2 Metabolism of DOF and DOE by SRD5A 54
5.2.2.1 Accurate mass determination of 11OHPdione and 11KPdione 54
5.2.2.2 UHPLC-MS/MS analyses of steroid metabolites 56
5.2.2.3 The conversion of C_{21} steroids by SRD5A in HEK293 cells 59
5.2.3 The conversion of 5α-reduced C_{21} steroids, 11OHPdione and 11KPdione by AKR1C2 in HEK293 cells 63
5.2.3.1 Accurate mass determination of 11OHPdiol and 11KPdiol 63
5.2.3.2 UHPLC-MS/MS analyses of steroid metabolites 65
5.2.4 DOF metabolism in cell models 66
5.2.4.1 UPC²-MS/MS analyses of C11-oxy C_{21} steroids 66
5.2.4.2 DOF conversion in PNT2, PC3 and LNCaP cells 70
5.3 Discussion 72

CHAPTER 6 CONCLUSION AND FUTURE PERSPECTIVES 75

BIBLIOGRAPHY 79

LIST OF FIGURES

Figure 2.1 Microscopic overview of the adrenal gland cross-section. The adrenal comprises the cortex and medulla, with the adrenal cortex further sub-divided into three zones. The three zones are characterised by the zone-specific expression of steroidogenic enzymes. 5

Figure 2.2 Basic structure of cholesterol that serves as precursor molecule for all steroid hormones. Carbon atoms numbered according to the standard convention, A and B ring indicated. 8

Figure 2.3 Adrenal steroidogenic pathway representing zone-specific enzyme expression and steroid secretion. Enzymes in dashed boxes display limited activity towards the substrate. 9

Figure 2.4 Steroidogenic pathways in 21OHD patients. P4 and 17OHP4 are shunted towards the CYP11B and 11βHSD enzymes producing C11-oxy C_{21} steroid hormones. Enzymes in dashed boxes have limited activity towards the substrate or requires full elucidation. 16

Figure 3.1 Conventional C_{19} pathway. Red arrows indicate pathway in which circulating T serves as a direct precursor for DHT production. Bold, underlined steroid metabolites are substrates for UGT2B enzymes. 21

Figure 3.2 Alternative 5α-dione pathway. Red arrows indicate the pathway that bypasses T in DHT production. Bold and underlined steroid metabolites indicate substrates for conjugation by UGT2B enzymes. 22

Figure 3.3 11OHA4 pathway. The conversion of the abundant adrenal steroid metabolite, 11OHA4, to more potent androgens. Bold underlined steroids can be conjugated by UGT2B enzymes. 23

Figure 3.4 Backdoor pathway (red arrows) indicate the production of DHT proceeding via the metabolism of 17OHP4, bypassing the conventional adrenal androgen precursors. Underlined and bold steroid metabolites are potential substrates for UGT2B enzymes. 25

Figure 4.1 Biosynthesis of C11-oxy C_{21} steroids in the 21OHD adrenal due to increased P4 and 17OHP4 concentrations. Enzymes conversions indicated in dashed boxes has not yet been confirmed. 31

Figure 4.2 MS spectra obtained for (A) 11OHP4 ($[M+H]^+$ *m/z* 331.21) and (B) DOF ($[M+H]^+$ *m/z* 347.4). Data was acquired from *m/z* 150-600. 36

Figure 4.3 UHPLC-MS/MS chromatographic separation of eight steroid metabolites. Structures and retention times of steroids (shaded peaks) are indicated on each chromatogram. Chromatograms were generated during a 5 µL injection of an extracted 2 µg/mL steroid standard using MRM. 38

Figure 4.4 Overlaid UHPLC-MS/MS chromatogram of P4, 11OHP4, 17OHP4, and DOF. Retention times are indicated on the chromatogram with P4 eluting at 4.20 minutes, 11OHP4 at 3.20 minutes, 17OHP4 at 3.39 minutes and DOF at 2.36 minutes. 39

Figure 4.5 Metabolism of 1 µM P4 by CYP11B1 (A) and CYP11B2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM. 40

Figure 4.6 Metabolism of 1µM 17OHP4 by CYP11B1 (A) and CYP11B2 (B) in transiently transfected HEK293 cells over 24 hours. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM. 40

Figure 4.7 Metabolism of 1µM A4 by the CYP11B isozymes in transiently transfected HEK293 cells after 24 hours. Results represented as the mean ±SEM. 0h, sample aliquot collected at the start of the assay. 41

Figure 4.8 Metabolism of 1µM P4 (A) and 17OHP4 (B) in HEK293 cells after 24 hours. Results represented as the mean ±SEM. 0h, sample aliquot collected at the start of the assay; pCIneo, HEK293 cells transfected with vector without cDNA (negative control). 41

Figure 4.9 Comparison of the catalytic activity of the CYP11B isozymes towards 1µM P4 (A) and 17OHP4 (B). An unpaired t-test was performed and statistical significance was confirmed with a $P < 0.05$. 42

Figure 4.10 Comparison of the metabolism of 1µM P4 and 17OHP4 by CYP11B1 (A) and CYP11B2 (B). Statistical significance was determined using an unpaired t-test, with significance confirmed for $P < 0.05$. 42

Figure 5.1 Production of DHT in the backdoor pathway (red arrows). The proposed metabolism of DOF and DOE and the biosynthesis of 11OHDHT and 11KDHT (boxed steroids) are indicated in the shaded area. 48

Figure 5.2 MS spectra of (A) 11OHPdione ($[M+H]^+$ *m/z* 349.4) and (B) 11KPdione ($[M+H]^+$ *m/z* 347.4). Acquired mass range was scanned across *m/z* 150-600. 55

Figure 5.3 UHPLC-MS/MS chromatographic separation of fourteen steroids. The structure and retention time of steroids (shaded peak) are indicated on the chromatograms. Chromatograms were generated using MRM mode (5 µL injection volume of the standard 2.5 ng/µL). Peaks were generated from the highest product in conversion assays for steroids without available standards. 58

Figure 5.4 Overlaid UHPLC-MS/MS chromatogram of SRD5A substrates and products in MRM mode. 59

Figure 5.5 Metabolism of 5 µM P4 by SRD5A1 (A) and SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM. 59

Figure 5.6 Metabolism of 5 µM 17OHP4 by SRD5A1 (A) and SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM. 60

Figure 5.7 Metabolism of 5 µM DOF by SRD5A1 (A) or SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM. 60

Figure 5.8 Metabolism of 5 µM DOE by SRD5A1 (A) or SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM. 61

Figure 5.9 Comparison of the catalytic activity of SRD5A1 (A) and SRD5A2 (B) with P4, 17OHP4, DOF and DOE in HEK293 cells. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM. 61

Figure 5.10 Comparison of the catalytic activity of SRD5A1 and SRD5A2 towards P4 (A), 17OHP4 (B), DOF (C) and DOE (D) in HEK293 cells. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM. 62

Figure 5.11 Metabolism of 1 µM T by the SRD5A isozymes in HEK293 cells after 12 hours. Results represent two independent experiments, performed in triplicate represented as the mean ±SEM. 0h, sample aliquot collected at start of the assay. 62

Figure 5.12 Metabolism of 5 µM of P4 (A), 17OHP4 (B), DOF (C) and DOE (D) in HEK293 cells after 12 hours. Results represent two independent experiments performed in triplicate, illustrated as the mean ±SEM. 0h, sample aliquot collected at start of the assay; pCIneo, HEK293 cells transfected with vector without cDNA (negative control); UT, untransfected HEK293, (control, endogenous enzyme expression). 63

Figure 5.13 MS spectra of (A) 11OHPdiol ($[M+H]^+$ *m/z* 351.2) and (B) 11KPdiol ($[M+H]^+$ *m/z* 349.2). Acquired mass range was scanned across *m/z* 150-600. 64

Figure 5.14 UHPLC-MS/MS analyses of DOF (A) and DOE (B) metabolites. Retention times are noted above each peak. 66

Figure 5.15 UPC^2-MS/MS chromatographic separation of 16 steroids. The structure and retention times of steroids (shaded area) are indicated on the chromatograms. Chromatographic profiles were generated using MRM mode (2 µL injection of standard, 1 µg/mL). 69

Figure 5.16 The metabolism of 1 µM DOF in PC3, PNT2 and LNCaP cell models. Steroid concentrations at 0 hour (A) and at 24 hours (B). 70

Figure 5.17 Metabolism of 1 µM DOF in PNT2 (A), PC3 (B) and LNCaP (C) cells after 24 hours. Results represent triplicate values as mean ±SEM. Results are depicted in µM concentrations and as response in the case of steroids for which no standards were available. 71

Figure 5.18 Metabolism 1 µM 17OHP4 in PNT2 (A), PC3 (B) and LNCaP (C) cells after 24 hours. Results represent triplicate values as mean ±SEM. 72

LIST OF TABLES

Table 4.1 UHPLC solvent parameters for the chromatographic separation. 34
Table 4.2 Plasmid yield and purity. 35
Table 4.3 Accurate mass determination of 11OHP4 and DOF. UHPLC retention times, accurate masses (observed and calculated), formulae and fragment ions are specified. 36
Table 4.4 UHPLC-MS/MS parameters for the separation of eight steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown. 37

Table 5.1 UHPLC solvent parameters for the chromatographic separation. 53
Table 5.2 UPC^2 solvent parameters for the chromatographic separation. 53
Table 5.3 Plasma yields obtained from DNA purification 54
Table 5.4 Accurate mass determination of 11OHPdione and 11KPdione. Accurate masses (observed and calculated), formulae and fragment ions are depicted. 55
Table 5.5 UHPLC-MS/MS parameters for the separation of fourteen steroids. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown. 56
Table 5.6 Accurate mass determination of 11OHPdiol and 11KPdiol. Accurate masses (observed and calculated), formulae and fragment ions are depicted. 64
Table 5.7 UHPLC-MS/MS parameters for the separation of steroid metabolites for AKR1C2 conversions. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown. 65
Table 5.8 UPC^2-MS/MS parameters for the separation of steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown. 67

ABBREVIATIONS

General

21OHD	21-hydroxylase deficiency
ACTH	adrenocorticotropic hormone
ADX	adrenal ferredoxin
AKR	short-chain dehydrogenase/aldo-keto reductase
AngII	angiotensin type II
AR	androgen receptor
CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
CE	collision energy
CRH	corticotropin-releasing hormone
CRPC	castration resistant prostate cancer
CV	cone voltage
Cytb5	cytochrome b_5
DMEM	Dulbecco's Modified Eagle's Medium
ER	endoplasmic reticulum
ESI^+	positive electrospray ionisation
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GC-MS	gas chromatography-mass spectrometry
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HSD	hydroxysteroid dehydrogenase
IGF-II	insulin growth factor 2
IMM	inner mitochondrial membrane
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low-density lipoproteins
LH	luteinising hormone
MC2R	melanocortin receptor type 2
MR	mineralocorticoid receptor
MRM	multiple reaction monitoring
MS	mass spectrometry
MTBE	methyl tertiary-butyl ether
NC 21OHD	non-classical 21-hydroxylase deficiency/late onset 21-hydroxylase deficiency

OMM	outer mitochondrial membrane
P450	cytochrome P450
PR	progesterone receptor
RT	retention time
StAR	steroidogenic acute regulatory
SV 21OHD	simple virilising 21-hydroxylase deficiency
SW 21OHD	salt wasting 21-hydroxylase deficiency
TIC	total ion count
UDP	uridine diphosphoglucuronic acid
UHPLC-MS/MS	ultra-high performance liquid chromatography-tandem mass spectrometry
UPC^2-MS/MS	ultra-performance convergence chromatography-tandem mass spectrometry
QTMS	triple quadropole mass spectrometer
Q-tof-MS	quadropole time-of-flight mass spectrometer
zF	zona fasciculata
zG	zona glomerulosa
zR	zona reticularis

Enzymes

11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
17βHSD3	17β-hydroxysteroid dehydrogenase type 3
17βHSD6	17β-hydroxysteroid dehydrogenase type 6/ human oxidative 3αHSD (RL-HSD)
3βHSD2	3β-hydroxysteroid dehydrogenase type 2
AKR1C3	3α-hydroxysteroid dehydrogenase type 2/17β-hydroxysteroid dehydrogenase type 5
AKR1C2	3α-hydroxysteroid dehydrogenase type 3
CYP11A1	cytochrome P450 cholesterol side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	cytochrome P450 aldosterone synthase
CYP17A1	cytochrome P450 17α-hydroxylase/17, 20 lyase
CYP19A1	cytochrome P450 aromatase
CYP21A2	cytochrome P450 steroid 21-hydroxylase
FNR	ferredoxin reductase
POR	P450 oxidoreductase
SRD5A1	steroid 5α-reductase type 1
SRD5A2	steroid 5α-reductase type 2
SULT2A1	sulfotransferase
UGT	UDP-glucuronosyltransferase

Steroid hormones

11KA4	11-ketoandrostenedione
11KAdiol	11-ketoandrostanediol
11KAdione	11-ketoandrostanedione
11KAST	11-ketoandrosterone
11KDHT	11-ketodihydrotestosterone
11KP4	11-ketoprogesterone
11KPdiol	5α-pregnane-3α, 17α-diol-11, 20-dione
11KPdione	5α-pregnan-17α-ol-3, 11, 20-trione
11KT	11-ketotestosterone
11OHA4	11β-hydroxyandrostenedione
11OHAdiol	11β-hydroxyandrostanediol
11OHAdione	11β-hydroxyandrostanedione
11OHAST	11β-hydroxyandrosterone
11OHDHT	11β-hydroxydihydrotestosterone
11OHP4	11β-hydroxyprogesterone
11OHPdiol	5α-pregnan-3α, 11β, 17α-triol-20-one
11OHPdione	5α-pregnan-11β, 17α-diol-3, 20-dione
11OHT	11β-hydroxytestosterone
16OHP4	16α-hydroxyprogesterone
17OHP4	17α-hydroxyprogesterone
17OHP5	17α-hydroxypregnenolone
18OHCORT	18-hydroxycorticosterone
A4	androstenedione
Adiol	androstanediol
Adiol-G	androstanediol-glucuronide
Adione	androstanedione
ALDO	aldosterone
AlloP5	5α-pregnan-3α-ol-20-one
AST	androsterone
CORT	corticosterone
D2-T	Testosterone 1,2-D2, 98%
D7-11OHA4	4-androsten-11β-ol-3,17-dione 2,2,4,6,6,16,16-D7, 98%
D7-A4	4-androsten-3,17-dione 2,2,4,6,6,16,16-D7, 98%
D9-P4	Progesterone 2,2,4,6,6,17A,21,21,21-D9, 98%
DHEA	dehydroepiandrosterone
DHP	dihydroprogesterone

DHT	dihydrotestosterone
DHT-G	dihydrotestosterone-glucuronide
DOC	11-deoxycorticosterone
DOE	21-desoxycortisone
DOF	21-desoxycortisol
P4	progesterone
P5	pregnenolone
Pdiol	5α-pregnan-3α, 17α-diol-20-one
Pdione	5α-pregnan-17α-ol-3, 20-dione
T	testosterone
T-G	testosterone-glucuronide

CHAPTER 1

INTRODUCTION

21-hydroxylase deficiency (21OHD) is the most common form of congenital adrenal hyperplasia (CAH), attributed to a recessive inborn metabolic mutation in the gene that encodes cytochrome P450 steroid 21-hydroxylase (CYP21A2). The disease is divided into three subtypes based on the clinical symptoms which characterise the disorder, *viz.* classical salt-wasting 21OHD (SW 21OHD), classical simple virilising 21OHD (SV 21OHD) and non-classical 21OHD (NC 21OHD). The global incidence of the classical forms are reported to be up to 1/13 000 live births of which 75% are SW 21OHD, with the non-classical form reported to have a much higher prevalence of 1/1 000 live births (Nimkarn, Lin-Su, & New, 2011; Parsa & New, 2017). SW 21OHD is the most severe form of the disease, presenting with a total loss of function in CYP21A2 activity and if left untreated may lead to death within the first week of life due to an acute adrenal crisis (Christakoudi, Cowan, & Taylor, 2013).

CYP21A2 expression is adrenal specific, and it is an essential enzyme in adrenal steroidogenesis, allowing the biosynthesis of mineralocorticoids and glucocorticoids. In 21OHD the flux towards these steroid hormone branches are diminished and there is an increase in the flux towards adrenal androgen precursors together with increasing levels of the CYP21A2 substrates, progesterone (P4) and 17α-hydroxyprogesterone (17OHP4). The backdoor pathway presents an attractive alternative route for the downstream peripheral metabolism of P4 and 17OHP4 and has been suggested to play a significant role in 21OHD patients (Kamrath, Hochberg, Hartmann, Remer, & Wudy, 2012). Androgen excess is the hallmark of the disorder, presenting with a broad spectrum of clinical symptoms. As such, newborn screenings for 21OHD are employed using an adrenocorticotropic hormone (ACTH) stimulation test, identifying increased 17OHP4 and androstenedione (A4) levels. This is however not a satisfactory measure and high false-positive results are often reported. Furthermore, the measurement of 17OHP4 and A4 cannot distinguish between carriers of 21OHD and NC 21OHD patients. Recent studies has therefore started to include a broader spectrum of steroids during analyses, including 21-desoxycortisol (DOF), the C11-hydroxylated product of 17OHP4 (A. F. Turcu et al., 2015).

The biosynthesis of androgens and androgen precursors by the adrenal gland have been shown to have a major influence on downstream androgen profiles, since steroids produced in the adrenal are circulated to various peripheral tissue where they are further metabolized, thereby having a major impact on the cellular steroid profile and on proliferation. Changes in androgen metabolism are especially evident in female patients (Fernand Labrie, 1991; Longcope, 1986) and the symptoms of androgen excess are also more noticeable, especially in females presenting with ambiguous genetalia. The development of secondary diseases such as polycystic ovary syndrome (PCOS) and testicular adrenal rest tumors (TART) have

furthermore been linked to the androgen excess in 21OHD (Auchus, 2015; Aycan, Baş, Cetinkaya, Yilmaz Agladioglu, & Tiryaki, 2013). Downstream metabolism of adrenal androgen precursors can therefore not be overlooked, especially when taking into account the prominent role of C11-oxy steroids in clinical conditions, such as prostate cancer (A. C. Swart & Storbeck, 2015). The biosynthesis of C11-oxy steroids is catalysed by the adrenal specific CYP11B isozymes, and downstream metabolism of C11-oxy steroids highlights the adrenal contribution to androgen production. In support of this, recent studies have investigated the metabolism of P4 and 17OHP4 in TART cells, a cell model for 21OHD, and have identified the presence of 11β-hydroxyprogesterone (11OHP4) and DOF (A. F. Turcu et al., 2015), indicative of possible 11β-hydroxylation of P4 and 17OHP4 in these cells.

In androgen dependent diseases the major culprit towards disease progression is dihydrotestosterone (DHT). Recent identification of additional steroid sources and the knowledge of tissue specific expression of steroidogenic enzymes led to the unravelling of alternative pathways that lead to the production of DHT. In addition, the biosynthesis of 11β-hydroxydihydrotestosterone (11OHDHT) and 11-ketodihydrotestosterone (11KDHT) has been demonstrated via the metabolism of the adrenal androgen precursor, 11β-hydroxyandrostenedione (11OHA4) in the aptly termed 11OHA4 pathway (Bloem et al., 2015; Storbeck et al., 2013). 11OHDHT and 11KDHT has since been reported to be potent androgenic metabolites, able to activate the androgen receptor (AR) at levels comparable to testosterone (T) and DHT, respectively at 1nM (A. C. Swart & Storbeck, 2015) and with all C11-oxy metabolites, with the exception of 11OHA4, comparable to DHT at 10 nM (Bloem et al., 2015). We therefore hypothesised that the adrenal may contribute to the biosynthesis of C11-oxy C_{21} steroids, specifically from P4 and 17OHP4, which are detected at high levels in 21OHD. We further hypothesised that the metabolism of these C11-oxy C_{21} steroids, catalysed by the enzymes in the backdoor pathway, may contribute to C11-oxy steroid metabolites, specifically the production of potent androgens such as 11OHDHT and 11KDHT.

This study will first provide a detailed overview of adrenal steroidogenesis, presented in chapter 2. Special focus is placed on the deviations from normal adrenal steroidogenesis in the 21OHD adrenal, focussing on the possible downstream metabolism of P4 and 17OHP4 and the increased androgen precursors in the 21OHD adrenal. Chapter 3 provides an overview of the pathways in androgen responsive tissue that contribute to the production of potent androgen metabolites. The obligatory enzymes in the activation of androgen precursors in the backdoor pathway are discussed.

Chapter 4 and 5 investigate the biosynthesis and metabolism of C11-oxy C_{21} steroids, in an attempt to address the following aims:

1. To determine the catalytic activity of CYP11B1 and CYP11B2 towards P4 and 17OHP4
2. To determine the downstream metabolism of DOF and DOE catalysed by the first enzyme in the backdoor pathway, *viz.* SRD5A

3. To determine the conversion of 11OHPdione and 11KPdione catalysed by AKR1C2
4. To investigate the metabolism of DOF by endogenous enzymes expressed in normal and prostate cancer cell models – PNT2, PC3 and LNCaP

Chapter 4 presents the investigation into the biosynthesis of C11-oxy C_{21} steroids, determining the catalytic activity of the CYP11B isozymes towards P4 and 17OHP4. In chapter 5 the focus shifts towards the metabolism of C11-oxy C_{21} steroids, presenting the conversion of DOF and 21-desoxycortisone (DOE) catalysed by the SRD5A isozymes and AKR1C2. The metabolism of DOF is presented in the cell models PNT2, PC3 and LNCaP. In chapter 6 the most important findings are summarised and discussed, providing an integrated view of the possible role of C11-oxy C_{21} steroids in 21OHD.

CHAPTER 2

ADRENAL STEROIDOGENSIS

2.1 Introduction

Adrenal steroid hormones play a crucial role in normal human physiology, regulating various important metabolic processes. Cholesterol serves as precursor for all steroid hormones and is converted to biologically active steroid hormones by a complex system of enzymes in a process known as steroidogenesis. The adrenal expresses numerous steroidogenic enzymes with steroidogenesis taking place within the adrenal cortex. Adrenal steroid hormones can be classified into three distinct classes based on their influence on and regulation of important biological processes, namely the mineralocorticoids, glucocorticoids and the adrenal androgen precursors. Mineralocorticoids are responsible for the regulation of the electrolyte and water balance; glucocorticoids control the metabolism of carbohydrates, proteins and lipids, as well as the immune and stress responses; and adrenal androgen precursors are involved in sexual growth and development. Abnormalities in hormone function and secretion has been linked to a broad spectrum of clinical conditions. In this chapter adrenal steroidogenesis will be discussed, focussing on different enzymes catalysing the production of mineralocorticoids, glucocorticoids and adrenal androgen precursors. Special attention will be placed on 21OHD and how defects in the 21-hydroxylase enzyme may influence the downstream metabolism of cholesterol in the adrenal, leading to an increased production of adrenal androgen precursors.

2.2 Overview of the adrenal gland

The adrenal glands are situated on the medial side of each kidney and are involved in the production of steroid hormones within the endocrine system. The gland is anatomically divided into two distinct endocrine tissues types with separate features. A layer of connective tissue, known as the capsule, encloses the adrenal, serving a protective role. Directly beneath the capsule lies the adrenal cortex in which the adrenal steroid hormones are produced. These hormones are derived from the metabolism of cholesterol via a complex system of enzyme catalysed reactions. Even though these steroid hormones are structurally closely related, they vary with regard to their specificity for steroid receptors. The adrenal medulla is located within the centre of the gland, surrounded by the adrenal cortex that comprises roughly 85% of the adrenal (Mihai, 2011). The medulla secretes the catecholamines, norepinephrine and epinephrine (adrenalin), with the latter involved in the fight-or-flight reaction (Hardy & Cooper, 2010).

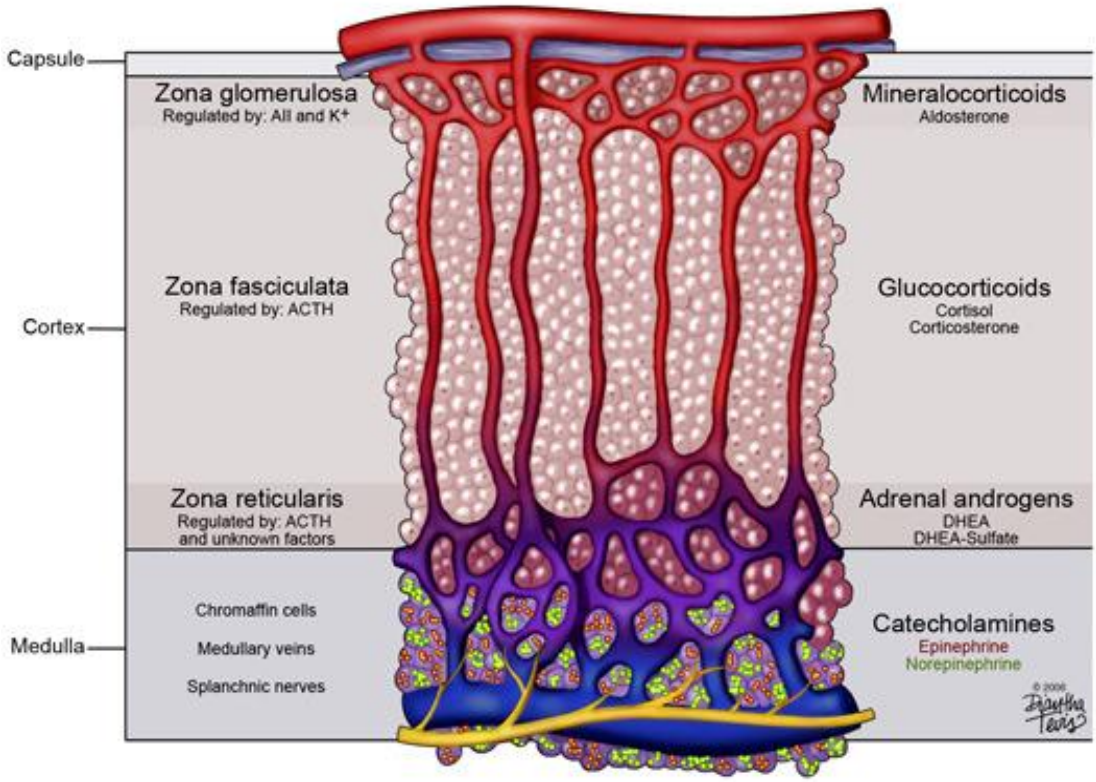


Figure 2.1 Microscopic overview of the adrenal gland cross-section. The adrenal comprises the cortex and medulla, with the adrenal cortex further sub-divided into three zones. The three zones are characterised by the zone-specific expression of steroidogenic enzymes.[1]

The adrenal cortex is further divided into three zones that are functionally distinct based on the expression of steroidogenic enzymes and co-factors within each region. This division leads to zone specific secretion of steroid hormones. The zona glomerulosa (zG) is situated directly beneath the capsule, forming the outermost layer and comprises roughly 15% of the adrenal cortex volume. This zone secretes mineralocorticoids, most prominently aldosterone (ALDO), under the regulation of the renin-angiotensin-aldosterone system, independent from ACTH. Three features are regulated by this system, the expression of angiotensin II (AngII) receptors, the expression of cytochrome P450 aldosterone synthase (CYP11B2) and lastly the absence of cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17A1) expression. The zona fasciculata (zF) forms the middle layer of the cortex and comprises almost 75% of the cortex. The main function of this zone is the secretion of glucocorticoids. The zF fails to express CYP11B2 or AngII receptors, but rather express melanocortin receptor type 2 (MC2R), the ACTH receptor, together with

[1] http://www.anatomy-diagram.info/adrenal-glands-diagram-structure/adrenal-gland-cortex/

[2] For the analyses of AKR1C2 conversion desolvation gas flow was set to 750 L/hour.

cytochrome P450 11β-hydroxylase (CYP11B1), thus losing the ability to produce ALDO, while retaining cortisol production capabilities. Both the zG and zF express CYP21A2 with the zF also expressing CYP17A1, allowing the biosynthesis of the glucocorticoid, cortisol, which regulates carbohydrate, protein and fat metabolism and immune processes. The zF however expresses negligible levels of cytochrome b_5 (cytb5), and as a result 17, 20-lyase activity by CYP17A1 has rarely been observed within this zone. Cortisol together with corticosterone (CORT) are produced under ACTH regulation, together with small amounts of dehydroepiandrosterone (DHEA). The zona reticularis (zR) encloses the medulla, forming the innermost part of the cortex. This zone expresses MC2R, but limited amounts of CYP21A2 and CYP11B1 and therefore produces only low levels of cortisol. The high expression levels of cytb5 in this zone together with CYP17A1, maximises the 17, 20-lyases activity of CYP17A1 leading to the formation of DHEA. Most of the DHEA produced is sulfonated by sulfotransferase (SULT2A1), limiting the precursors channelled into the adrenal androgen pathway and thus downstream production of androgen precursors. DHEA is converted to A4 through the activity of the enzyme 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), followed by the conversion to T, through the action of 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5), better known as AKR1C3 which is expressed at low levels in the adrenal. In addition, A4 and T are also converted to 11OHA4 and 11β-hydroxytestosterone (11OHT) through the action of the CYP11B isozymes. The zR is therefore responsible for the biosynthesis of adrenal androgen precursors, together with low levels of T (Hardy & Cooper, 2010; Miller & Auchus, 2011; A. C. Swart et al., 2013).

2.3 Regulation of adrenal steroid production

The principle mineralocorticoid, ALDO, and glucocorticoid, cortisol, together with the adrenal androgen precursors DHEA, 11OHA4 and A4 are secreted into circulation to exert physiological effects or undergo further downstream metabolism in peripheral target tissue (Rege et al., 2013; Turcu, Smith, Auchus, & Rainey, 2014). As previously stated ALDO production is regulated by the renin-angiotensin-aldosterone system, specifically AngII and circulating potassium levels, while glucocorticoid and adrenal androgen production are regulated by the hypothalamic-pituitary-adrenal (HPA) axis. AngII regulates ALDO secretion through the inhibition of renin and the stimulation of the conversion of cholesterol to pregnenolone (P5) and CORT to ALDO within the zG. The hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary gland to secrete ACTH, ultimately leading to the stimulation of the adrenal cortex and cortisol production. The pituitary gland also produces follicle-stimulating hormone (FSH) and luteinising hormone (LH) that regulate the gonadal hormone secretion in the hypothalamic-pituitary-gonadal (HPG) axis. Both of these axes are regulated through a negative feedback mechanism, whereby hormones produced in the adrenal/gonadal axis will inhibit the further secretion of stimulating hormones by the hypothalamus (long term) and pituitary (rapid) glands. ACTH secretion is therefore positively regulated by CRH and negatively by glucocorticoids, with the glucocorticoids exerting effects on the hypothalamus, pituitary and hippocampus (Hardy & Cooper, 2010; Mihai, 2011; Williams, 1988). No negative feedback mechanism by the adrenal androgens precursors on

the HPA have been reported to date while estrogens and excess adrenal androgens may have a negative feedback on the HPG (Mouritsen, Juul, & Jørgensen, 2010; Nimkarn & Lin-su, 2016; Sharifi & Auchus, 2012; Turcu et al., 2014; Vihko et al., 2004).

Steroidogenic cells store limited amounts of steroid hormones, therefore ACTH plays a pivotal role in steroidogenesis in not only maintaining the steroidogenic machinery but also by promoting cell growth. ACTH stimulates the production of intracellular cyclic adenosine monophosphate (cAMP) that supports adrenal growth, which in turn, stimulates the uptake of plasma low-density lipoproteins (LDL) cholesterol. This increases the availability of free cholesterol for steroid hormone biosynthesis, together with the biosynthesis of insulin growth factor 2 (IGF-II), basic and epidermal growth factor, thus defining the amount of steroidogenic tissue (Bose, Sugawara, Strauss, & Miller, 1996; Mesiano & Jaffe, 1997; Mesiano, Mellon, Gospodarowicz, Di Blasio, & Jaffe, 1991). ACTH furthermore governs the steroidogenic machinery within the cell by promoting the transcription of genes encoding the steroidogenic enzymes and co-factor proteins. Steroidogenic acute regulatory (StAR) gene transcription phosphorylation is also regulated by ACTH causing the rapid flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Arakane et al., 1996; Lin et al., 1995). This is a crucial initial step, considering the enzyme catalysing the rate limiting step in steroidogenesis, namely cytochrome P450 cholesterol side-chain cleavage (CYP11A1), is located on the IMM. CYP11A1 converts cholesterol to P5, an universal substrate for steroidogenic pathways (Miller, 2013; Miller & Auchus, 2011).

2.4 Biosynthesis of adrenal steroid hormones from cholesterol

The metabolism of cholesterol to more active steroid hormones, is a major part of the human endocrine system. Steroidogenesis takes place in a cell specific manner with the distribution and expression of steroidogenic enzymes determining the steroidogenic capacity of the cell. In mammals, cholesterol, in the form of plasma LDL, is derived mainly from dietary intake. The human adrenal can however also synthesise cholesterol *de novo* from acetate, mainly in the endoplasmic reticulum (ER) (Mason & Rainey, 1987). In the adrenal and gonads the rapid movement of cholesterol from the OMM to the IMM is regulated by the StAR protein (Lin et al., 1995). StAR acts exclusively in steroidogenic cells on the OMM, with its activity influenced by the cellular location and interaction with the OMM. StAR is inactive when located in the mitochondrial intermembranous space or matrix (Arakane et al., 1996). Cholesterol (Figure 2.2) serves as precursor molecule for all downstream steroidogenic enzyme catalysed reactions. In the absence of StAR, steroid hormones are synthesised in steroidogenic cells at 14% of its normal rate, highlighting the importance of StAR in the maintenance of steroidogenesis (Bose et al., 1996).

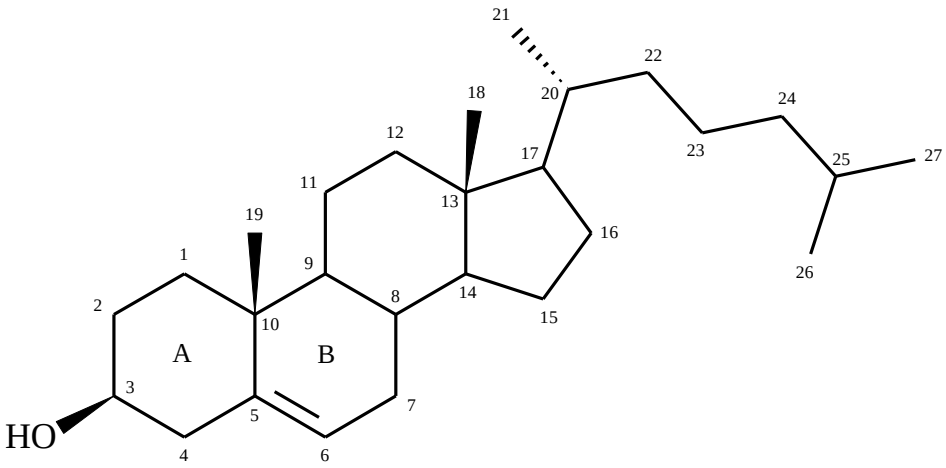


Figure 2.2 Basic structure of cholesterol that serves as precursor molecule for all steroid hormones. Carbon atoms numbered according to the standard convention, A and B ring indicated.

Once cholesterol has been transported to the IMM the first step in steroidogenesis can take place, namely the conversion of cholesterol to P5 catalysed by CYP11A1. This is a rate-limiting step and the expression of CYP11A1 often dictates the steroidogenic capacity of a cell. Cholesterol steroid derivatives are either C_{21} steroids (such as ALDO or cortisol produced in the mineralocorticoid and glucocorticoid pathways) or C_{19} steroid molecules (adrenal androgen precursors such as DHEA and A4). P5 is a Δ^5 steroid, together with 17OHP4 and DHEA, all containing a double bond in the B-ring and hydroxyl-group on C3. The subsequent conversion of these Δ^5 steroids by 3βHSD2 leads to the isomerisation of the double bond to the A-ring and translates the C3 hydroxyl-group to a keto-group, yielding the Δ^4 steroids, mostly more active than their Δ^5 steroid equivalents (Mihai, 2011; Miller & Auchus, 2011).

2.5 Enzymes catalysing the biosynthesis of adrenal steroid hormones

Steroidogenic enzymes are grouped into two classes based on their catalytic ability. The first enzyme class, cytochrome P450 (P450) enzymes, occurs as either type 1 or type 2 based on their intra-cellular location and mechanism of electron acceptance. The second enzyme class is also subdivided based on their structure into hydroxysteroid dehydrogenase (HSD) or short-chain dehydrogenase/aldo-keto reductase (AKR) families (Miller & Auchus, 2011).

Within the adrenal steroidogenic pathway (Figure 2.3), six P450 enzymes are involved together with several HSD enzymes organised in the intricate web of steroid hormone production. CYP11A1, CYP11B1 and CYP11B2 are located in the mitochondria and characterised as P450 type 1 enzymes, receiving their electrons from ferredoxin and ferredoxin reductase (FNR). FNR is a mitochondrial flavoprotein widely expressed in human tissue and together with ferredoxin forms an electron shuttle for all mitochondrial P450 enzymes (Miller & Auchus, 2011). FNR first receives an electron from NADPH, forming a complex with ferredoxin to allow electron transfer before dissociating, allowing the reduced ferredoxin to form a complex with the P450 enzyme (Lambeth, Seybert, & Kamin, 1979). Ferredoxin freely resides in the mitochondrial

matrix or loosely bound to the IMM. cAMP strongly induced the expression of ferredoxin within steroidogenic tissue (Brentano, Black, Lin, & Miller, 1992; Miller & Auchus, 2011). The remaining type 2 enzymes, CYP21A2, CYP17A1 and cytochrome P450 aromatase (CYP19A1) are located within the ER and receive electrons form P450 oxidoreductase (POR). POR receives two electrons from NADPH, and transfers them one at a time to the P450 enzyme (Miller, 2005; Miller & Auchus, 2011).

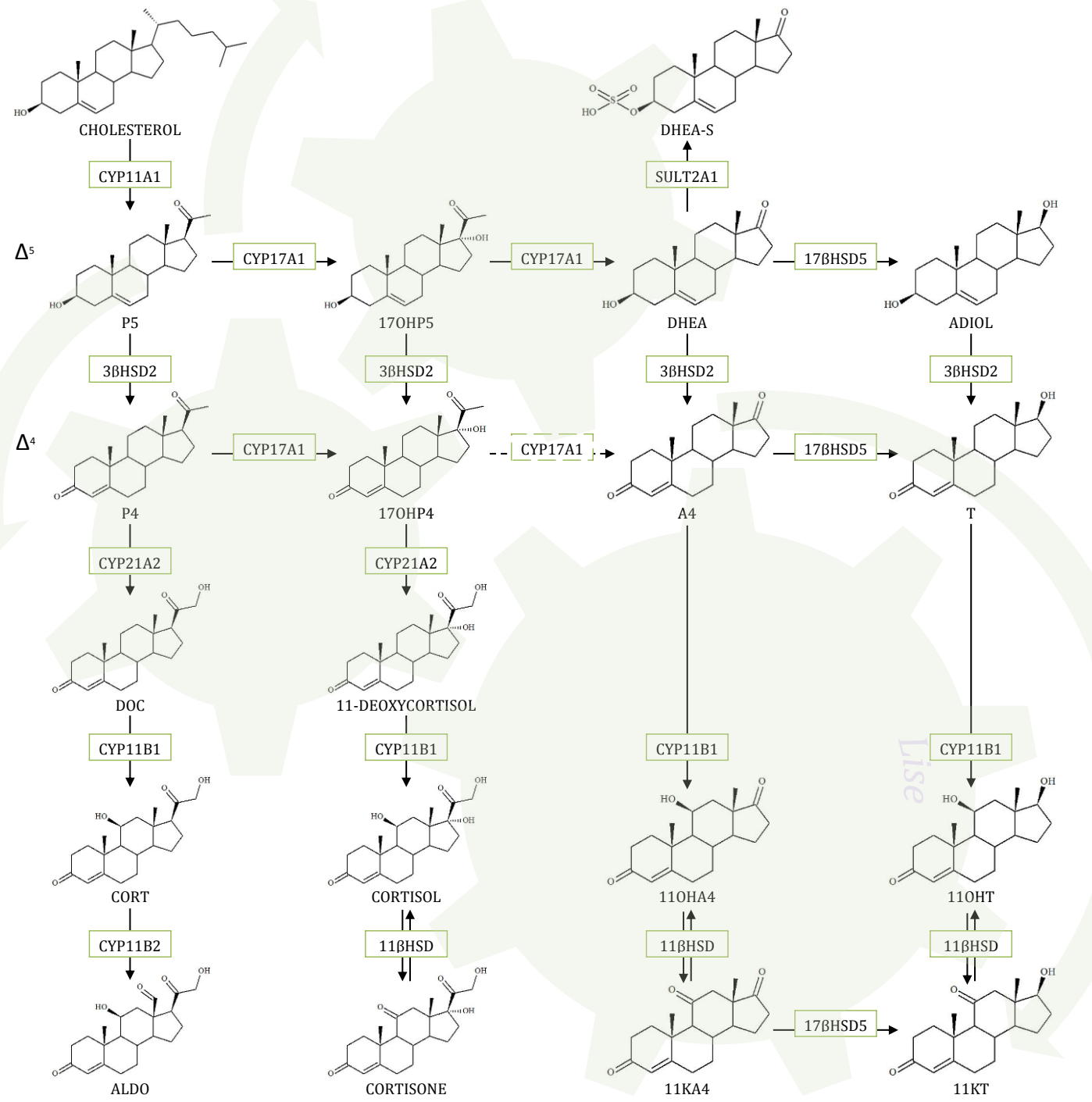


Figure 2.3 Adrenal steroidogenic pathway representing zone-specific enzyme expression and steroid secretion. Enzymes in dashed boxes display limited activity towards the substrate.

The adrenal express several 17β-hydroxysteroid hydrogenase (17βHSD) types, albeit at low levels, with the most crucial the expression of 17βHSD5 or AKR1C3, that catalyses the conversion of A4 to T (Matsuura et al., 1998; Nakamura, Hornsby, et al., 2009). The type 1 and 2 11β-hydroxysteroid dehydrogenases (11βHSD1 and 11βHSD2) are also expressed in negligible amounts while high levels of 3βHSD2 are expressed. Negligible expression of steroid 5α-reductase type 2 (SRD5A2) in the adrenal has also been reported using microarray analyses (Rege et al., 2013).

2.5.1. The cytochrome P450 enzymes

NAD(P)H acts as electron donor for all P450 enzymes, receiving electrons to their heme centre. In this reduced complex they have the unique ability to absorb light at 450 nm, hence the name P450. These enzymes mediate irreversible hydroxylation and carbon-carbon cleavage reactions, and as previously mentioned, six different P450 enzymes are involved in adrenal steroidogenesis catalysing these reactions (Miller & Auchus, 2011).

Since CYP11A1 catalyses the initial, rate-limiting step in cholesterol metabolism, the levels at which this enzyme are expressed in a cell dictates the steroidogenic capacity of the cell. CYP11A1 catalyse three successive reactions, the 20- and 22-hydroxylation followed by the cleavage of the 20, 22-bonds on cholesterol, leading to the production of P5 and isocaproaldehyde (Shikita & Hall, 1974). The expression of CYP11A1 therefore permits the *de novo* biosynthesis of steroids with the reaction catalysed being tissue-specific and hormonally regulated. CYP11A1 is ubiquitously expressed in all of the adrenal zones at high levels, induced by cAMP in the adrenal zF and zR, ovaries and testis and by the calcium/protein kinase C system in the adrenal zG (John, John, Boggaram, Simpson, & Waterman, 1986; Mellon & Vaisse, 1989; Moore, Brentano, & Miller, 1990). CYP11A1 is active within the mitochondria, receiving electrons from its redox partners, close to the site of cholesterol delivery and is the only enzyme that can form substantial amounts of P5 from cholesterol, and as such is responsible for the quantitative levels of steroid biosynthesis (Miller, 2013; Miller & Auchus, 2011).

P5 is transported from the mitochondria to the ER where it is either catalysed by both 3βHSD2 or CYP17A1 (Figure 2.3). The microsomal enzyme CYP17A1, which is encoded by a single gene expressed in the adrenals and gonads, exhibits both 17α-hydroxylase and 17, 20–lyase activity. The dual enzyme activity of CYP17A1 is at a key branch point within steroid hormone biosynthesis and has the potential to differentiate between the glucocorticoid and adrenal androgen production, playing a major role in the metabolism of Δ^5 steroids. CYP17A1 is therefore a crucial player in steroid hormone biosynthesis and the expression of this enzyme together with other steroidogenic enzymes, such as CYP21A2, within the specific cortical zones, dictate the biosynthesis of the different groups of steroid hormones. No CYP17A1 activity is present in the zG and P5 is metabolised by enzymes in the mineralocorticoid pathway, ultimately leading to the production of ALDO (Suzuki et al., 2000). Within the zF the hydroxylase activity of

CYP17A1 is the dominant reaction, since the lyase activity is restricted due to limited cytb5 expression (Mapes, Corbin, Tarantal, & Conley, 1999). P5 is hydroxylated at C17, forming 17α-hydroxypregnenolone (17OHP5), a precursor in glucocorticoid production. Similarly, P4 serves as a substrate for CYP17A1 activity, forming 17OHP4. Additionally, CYP17A1 has the ability to 16α-hydroxylate P4, forming 16α-hydroxyprogesterone (16OHP4), a reaction not observed with P5 (P. Swart, Swart, Waterman, Estabrook, & Mason, 1993). High CYP17A1 and cytb5 expression promotes the dual enzyme activity of CYP17A1 in the adrenal zR, allowing the biosynthesis of adrenal androgen precursors. P5 serves as substrate for the dual activity of CYP17A1, yielding DHEA, with 17OHP5 as intermediate. The hydroxylation of P4 and P5 is catalysed with equal efficiency, but major differences are found in the activity towards the resulting hydroxylated Δ^5 and Δ^4 products. The 17, 20-lyase activity shows favour towards 17OHP5 over 17OHP4, leading to a more efficient catalytic activity in the Δ^5-pathway compared to the Δ^4-pathway, yielding DHEA rather than A4. The catalytic activity of CYP17A1 towards the Δ^5 steroids thus forms the principle pathway towards C_{19} steroid hormones (Flück, Miller, & Auchus, 2003; Imai, Globerman, Gertner, Kagawa, & Waterman, 1993; Lee-Robichaud, Wright, Akhtar, & Akhtar, 1995). POR mainly regulates the 17, 20-lyase activity through the electron transport of NADPH, and the availability of electrons determines whether both reactions can occur. The 17, 20-lyase activity of CYP17A1 may be increased through the presence of cytb5, however the catalytic activity never reaches the efficiency of the hydroxylation reaction and even with the addition of cytb5 in experimental assays the preference of the lyase activity for the Δ^5 substrates persists (Lee-Robichaud et al., 1995). Cytb5 is expressed mainly in the adrenal zR where it can interact with CYP17A1 to increase the 17, 20-lyase activity of this enzyme contributing to increased production of adrenal androgen precursors by this zone (Mapes et al., 1999). Cytb5 works in conjunction with POR, acting as an allosteric activator by promoting the interaction between CYP17A1 and POR in which the presence of cytb5 increases the lyase activity of CYP17A1 more than 10-fold (Katagiri, Kagawa, & Waterman, 1995).

The metabolism of Δ^4 steroid precursors in the glucocorticoid and mineralocorticoid pathways is dependent on catalytic conversion by CYP21A2. This enzyme's expression is most abundant in the adrenal within the zG and zF catalysing the hydroxylation reaction at C21 (Luu-The, Pelletier, & Labrie, 2005; Pezzi, Mathis, Rainey, & Carr, 2003). Both P4 and 17OHP4 act as a substrate for this enzyme forming 11-deoxycorticosterone (DOC) and 11-deoxycortisol respectively. CYP21A2 is located in the ER where it utilises POR as its sole electron donor (Kominami, Hara, Ogishima, & Takemori, 1984). A defect in this enzyme, known as 21OHD, has a major impact on adrenal steroidogenesis, causing the accumulation of the CYP21A2 substrates, P4 and 17OHP4, discussed later in more detail.

The CYP11B isozymes are involved in the further downstream conversion of the 21-hydroxylated steroids in the glucocorticoid and mineralocorticoid pathways. CYP11B1 is mainly expressed in the zF and to a lesser extent in the other zones where it catalyses 11β-hydroxylation reactions seen in the conversion of 11-deoxycortisol to cortisol, and DOC to CORT. CYP11B2 is mainly expressed in the adrenal zG where it

catalyses the 11β-hydroxylase, 18-hydroxylase and 18-methyl oxidase activities, converting DOC to ALDO, forming the intermediates CORT and 18-hydroxycorticosterone (18OHCORT) in the process (Kawamoto et al., 1992; Nishimoto et al., 2010; Pezzi et al., 2003). Recent studies in our laboratory have shown that A4 and T are also converted by both CYP11B isozymes to 11OHA4 and 11OHT (Swart et al., 2013). While ACTH regulates the expression of CYP11B1 via cAMP, the enzyme is suppressed by glucocorticoids, with CYP11B2 expression being regulated mainly by the renin-angiotensin-aldosterone system (Clyne et al., 1997). In addition, both isozymes are located on the IMM and as such they compete with CYP11A1 for electrons from ferredoxin and FNR (Chua et al., 1987).

2.5.2 The hydroxysteroid dehydrogenase enzymes

A broad spectrum of HSD enzymes are involved in steroidogenesis, including 3α- and 3βHSD, two 11βHSD isoforms and various 17βHSD enzymes. The family of HSD enzymes have the ability to either oxidise steroid substrates, using NAD^+ as co-factor, or reduce steroid substrates using NADPH. Even though HSD reactions are mechanistically reversible and typically bi-directional *in vitro*, a clear preference towards oxidation or reduction is found *in vivo*, based on the availability and concentration of co-factors and the enzymes relative affinity towards NAD(P)H and $NAD(P)^+$ (Miller & Auchus, 2011).

In the steroidogenic pathways in the adrenal, 3βHSD2 plays an important role at the branch point, in conjunction with CYP17A1 and catalyses the conversion of Δ^5 to Δ^4 steroids by two successive chemical transformations. The first step converts the hydroxyl group at C3 to a keto group, utilising NAD^+ to produce NADH in the process. The intermediary product remains tightly bound to the enzyme and the presence of NADH in the co-factor binding site activates the enzyme's second activity, *viz.* the isomerisation of the double bond from the B-ring to the A-ring, leading to the isomerisation of Δ^5 to Δ^4 steroids (Luu-The et al., 1991; Thomas, Frieden, Nash, & Strickler, 1995). 3βHSD2 is expressed in the adrenals and gonads, and is located in both the mitochondria and ER (Pezzi et al., 2003; Suzuki et al., 2000). This enzyme is mainly membrane-bound, associating with CYP11A1 on the IMM, competing with CYP17A1 for P5 as substrate. However, the intermitochondrial orientation of 3βHSD2 promotes the biosynthesis of P4 over 17OHP5, with products of the Δ^4-pathway strongly inhibiting 3βHSD2 activity (Thomas, Berko, Faustino, Myers, & Strickler, 1989). P5, 17OHP5, DHEA and androstanediol (Adiol) are all substrates for this enzyme, irreversibly catalysing the conversion to P4, 17OHP4, A4 and T respectively (Lee, Miller, & Auchus, 1999; Thomas et al., 1989). In the adrenal zG, high levels of 3βHSD2 activity are observed together with the lack of CYP17A1 expression. This zone specific expression allows the 3βHSD2 activity to take precedence, allowing the conversion of P5 to P4. Within the adrenal zF the expression of both 3βHSD2 and CYP17A1 allows the production of glucocorticoids, via the biosynthesis of the glucocorticoid precursor 17OHP4 (Suzuki et al., 2000). 3βHSD2 has a higher Km value than CYP17A1 for the Δ^5 steroids and therefore a lower affinity, favouring the production of steroids in the Δ^5-pathway over the Δ^4-pathway (Auchus, Lee, & Miller, 1998; Lee et al., 1999). 17OHP4 is thus mainly formed by the conversion of P5 to 17OHP5 by CYP17A1, followed by the conversion to 17OHP4 by 3βHSD2. In the zR

most of the DHEA produced is sulfonated by SULT2A1, observed at high levels within this zone, however DHEA can also be converted by 3βHSD2 yielding A4 (Nakamura, Gang, Suzuki, Sasano, & Rainey, 2009). The production of A4 happens primarily through the Δ^5 pathway rather than the Δ^4 pathway, since the lyase activity towards 17OHP4 is restricted (Auchus et al., 1998). Recent studies in our laboratory revealed that cytb5 augments 3βHSD2 in a substrate and specie specific manner. Cytb5 acts as an allosteric regulator by increasing the affinity of 3βHSD2 for NAD^+ and thereby the enzyme's overall catalytic efficiency. The greatest effect of cytb5 was observed with P5 as substrate and to lesser extent DHEA, with limited effect towards 17OHP5 (Goosen, Storbeck, Swart, Conradie, & Swart, 2011; Goosen, Swart, Storbeck, & Swart, 2012). The potential interaction of cytb5 and 3βHSD2 was recently brought to the fore when evidence of cells at the interface between the zF and zR were shown to express high levels of both cytb5 and 3βHSD2 leading to increased A4 and T production (Turcu et al., 2016).

The 17βHSDs, also termed 17-oxidoreductases or 17-ketosteroid reductases, play a key role in androgen metabolism, and more than fifteen different subtypes have to date been reported (Luu-The, Bélanger, & Labrie, 2008; Peltoketo, Luu-The, Simard, & Adamski, 1999; Vihko et al., 2004, 2006). In the adrenal only type 2, 3 and 5 appear to play a role, with both 17βHSD2 and 17βHSD3 expressed in limited amounts (Rege et al., 2013), having a more prominent role in sex steroid metabolism in the gonads. Also catalysing the reduction of the C17 keto group, converting the C17 keto group to a hydroxyl-group as in the case of A4 to T, is 3α-hydroxysteroid dehydrogenase type 2 (AKR1C3), also known as 17βHSD5. Even though this enzyme exhibits poor catalytic activity towards A4, it has been suggested that AKR1C3, expressed within the adrenal zR, participates in adrenal T production (Nakamura, Hornsby, et al., 2009). The low levels of AKR1C3 reported in the adrenal can produce Adiol and T from DHEA and A4 respectively, although T formation may also be due to 3βHSD2 activity with Adiol as substrate.

Both 11βHSD1 and 11βHSD2 expression have been reported in the human adrenal (Mazzocchi et al., 1998; Rege et al., 2013; Ricketts et al., 1998). The type 1 enzyme catalyses the oxidation of cortisol to cortisone utilising $NADP^+$ as co-factor and can also catalyse the reverse reaction, converting cortisone to cortisol, utilising NADPH as co-factor. *In vivo* the reductive reaction takes precedence. 11βHSD2 exhibit only oxidative activity, catalysing the conversion of active cortisol to inactive cortisone (Stewart, 2003). Similarly CORT and 11-dehydrocorticosterone interconversion by these isozymes has been reported (White, Mune, & Agarwal, 1997). Since 11βHSD activity has been observed within the adrenal, both subtypes may contribute to the inactivation of cortisol, forming cortisone. Within the adrenal androgen pathway 11OHA4 and 11OHT have been identified as additional substrates for 11βHSD enzymes, allowing the biosynthesis of 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT) (Swart et al., 2013), although limited production thereof has been observed by the adrenal (Rege et al., 2013).

2.6 Adrenal steroidogenesis in 21OHD

CAH is a adrenal developmental disorder resulting in primary adrenal insufficiency (Miller & Auchus, 2011). This disease is an inherent metabolic disorder affecting the production of mineralocorticoids, glucocorticoids and adrenal androgen precursors in the adrenal due to an autosomal recessive gene. The most common form of CAH is a mutation in the gene encoding CYP21A2 that influences 21-hydroxylase activity and is responsible for up to 95% of CAH cases. As CYP21A2 catalyses the conversion of P4 to DOC and 17OHP4 to 11-deoxycortisol, it supplies the precursors for ALDO and cortisol production respectively. The clinical manifestation of this enzyme defect is known as 21OHD. This disorder has a general incidence of 1 in 14 200 patients and is reported to be the most common genetic endocrine disorder (Alonso-Fernández, 2016; Gonçalves et al., 2013). Depending on the specific mutation, the loss in 21-hydroxylase activity can be complete or partial and the severity of 21OHD loss correlates strongly with the clinical phenotype (Alonso-Fernández, 2016). This disease is characterised by low glucocorticoid production together with excessive adrenal androgen production, with or without mineralocorticoid insufficiency (Gonçalves et al., 2013) and is characterised by increased levels of 17OHP4, A4, DOF and to a lesser degree T, excreted in adult urine. In neonates this pattern is more complex, having a broad spectrum of different hydroxylated metabolites, with the balance between 11β-hydroxy and 11-keto steroids favouring the production of the oxidated keto form of the steroid (Christakoudi, Cowan, & Taylor, 2013).

Complete loss in 21-hydroxylase activity leads to the most severe form of the disease, known as SW 21OHD. Mutations with a 98% loss in activity results in virilisation, known as SV 21OHD and mutations leading to an 80-90% decrease in activity are referred to as NC 21OHD, also known as late onset 21OHD. NC 21OHD affects between 0.1-0.2% of the Causcasian population and does not present symptoms during early childhood, but can lead to excessive androgen levels, accelerated bone aging and adolescent and adult female hirsutism, irregular menstruation and infertility. SV 21OHD has a 25% incidence in all classical cases, and the effects of excessive androgen levels are already evident during childhood where newborn females may be born with ambiguous external genetalia. On the other hand, SW 21OHD is potentially life-threatening and if left untreated can lead to an acute adrenal crisis within the first three weeks after birth, leading to insufficient weight gain, vomiting, diarrhoea, dehydration, failure to thrive, lethargy, hypernatremia (due to 21OHD induced ALDO deficiency), hyperkalaemia and shock (Alonso-Fernández, 2016). Inadequate therapy can lead to early puberty, short stature and infertility due to hyperandrogenism, whereas a too high dosage treatment can lead to insulin resistance, osteoporosis and an increase body mass due to the effects of hypercortisolism (Gonçalves et al., 2013).

The HPA axis feedback system is mediated by circulating cortisol levels and the steroid's negative feedback on CRF and ACTH secretion. The block in cortisol production during 21OHD leads to increased CRH and ACTH production by the hypothalamus and pituitary in the absence of the negative regulatory feedback mechanism. These persistent elevated ACTH levels overstimulate the adrenal cortex in an attempt

to increase cortisol production, ultimately leading to hypertrophy of the adrenal cortex. The build-up of precursor molecules, especially 17OHP4, in pathways inhibited by the defective CYP21A2 enzyme, is directed towards the biosynthesis of adrenal products in pathways unaffected by the defect (Figure 2.4). 17OHP4 is shunted towards the intact androgen pathway that does not require CYP21A2, where the biosynthesis of precursors for androgen production can occur, stimulated by ACTH (Kamoun et al., 2014; Nimkarn et al., 2011; Nimkarn & New, 2009; Parsa & New, 2017; A. Turcu et al., 2014).

2.6.1 Adrenal androgen steroid production during 21OHD

CYP21A2 is a crucial enzyme in the biosynthesis of glucocorticoids and mineralocorticoids. A defect in this enzyme results in a shunt towards the production of adrenal androgen precursors. Most steroidogenic enzymes are unidirectional, and as such the build-up of products will not direct the flux back towards the substrate, thus allowing alternative metabolism (Miller & Auchus, 2011). In 21OHD the activities of 3βHSD2 and CYP17A1 play a key role in the metabolism of the steroid precursor, P5. As discussed previously, the expression and biological activity of these two enzymes within the three adrenal zones determine the key steroids produced within each branch. Depending on the severity of the defect in CYP21A2, production of the principle mineralocorticoid, ALDO, and the leading glucocorticoid, cortisol, may occur through limited CYP21A2 activity (Christakoudi, Cowan, Christakudis, & Taylor, 2013).

Considering the classical illustration of adrenal steroidogenesis (Figure 2.3) the block in steroidogenic pathways due to the absence of CYP21A2 activity will lead to the accumulation of P4, 17OHP4, A4 and DHEA, formed in the metabolism of P5 by 3βHSD2 and CYP17A1. The accumulation of 17OHP4 is characteristic of 21OHD and a key marker for this disease. It is possible that the steroids which accumulate will be subjected to downstream metabolism by the enzymes of the glucocorticoid and mineralocorticoid pathways, which include CYP11B1 and 11βHSD2. This would lead to both the production of C11-oxy C_{19} steroids, as well as that of potential C11-oxy C_{21} steroid metabolites within the adrenal (Figure 2.4). Metabolism of P4 in TART have been shown to yield 11OHP4, 16OHP4 and 17OHP4, while 17OHP4 yielded DOF (Turcu et al., 2015). With the demonstration of 11OHA4 being an adrenal precursor for more potent androgens such as 11OHT and 11KT (Storbeck et al., 2013), new interest has been placed on the role that CYP11B may play in the production of potent androgens. Investigations into the levels of C11-oxy C_{19} steroids have shown that 11OHA4, 11OHT, 11KA4 and 11KT were significantly higher in patients with 21OHD (Rege et al., 2013; Turcu et al., 2016). In addition, the biosynthesis of C11-oxy steroids presents additional substrates for the 11βHSD enzymes. The adrenal expresses 11βHSD1 and 11βHSD2 (Rege et al., 2013) and the metabolism of 11OHP4 and DOF, yielding 11-ketoprogesterone (11KP4) and DOE in the adrenal, as well as in peripheral tissue expressing these enzymes, is therefore plausible.

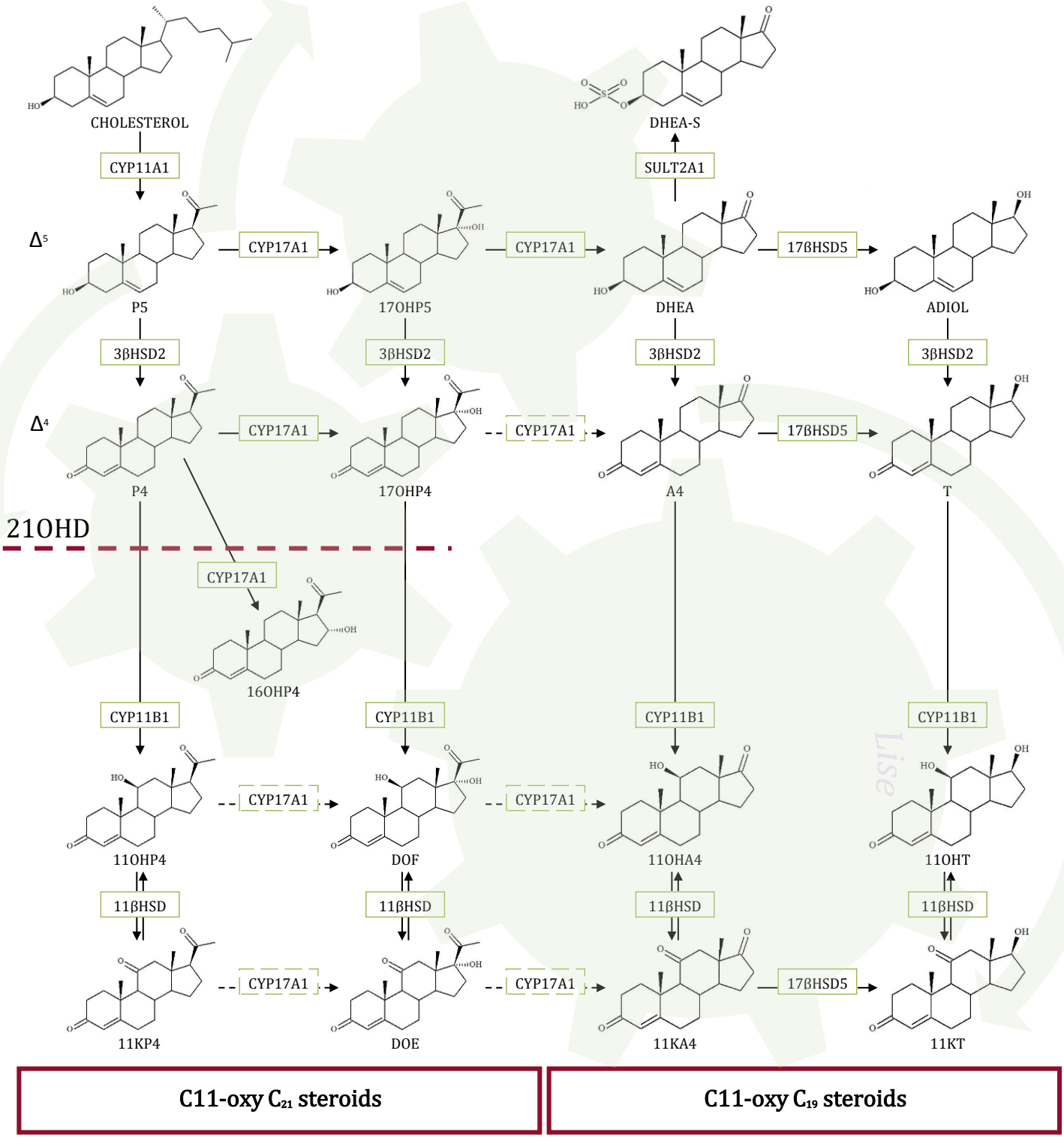


Figure 2.4 Steroidogenic pathways in 21OHD patients. P4 and 17OHP4 are shunted towards the CYP11B and 11βHSD enzymes producing C11-oxy C_{21} steroid hormones. Enzymes in dashed boxes have limited activity towards the substrate or require full elucidation.

2.7 Screening of 21OHD

Biochemical diagnoses of 21OHD is clinically confirmed by hormonal evaluation. 21OHD has been added to the list of inborn metabolic disorders to be screened at birth due to the high frequency and life-

threatening potential of this disorder, coupled to the fact that it can be effectively treated with cortisol replacement therapy (Alonso-Fernández, 2016). The gold standard for the screening of 21OHD is the ACTH stimulation test that measures the levels of 17OHP4 and A4 at baseline and 60 minutes after intravenous ACTH administration (Nimkarn et al., 2011; Wilson et al., 2007). This method is however ineffective since 17OHP4 levels are generally elevated in unaffected newborns, especially if they are born premature, ill or stressed. In healthy newborns, 17OHP4 levels will decrease drastically after birth, but has usually not yet reached normal levels before a sample is taken (within three days after birth), leading to substantial inconsistencies in results. Detectable overlaps in the analyses of 17OHP4 levels of newborn samples with and without 21OHD often lead to clinical misdiagnoses. Even in cases with high sensitivity, up to 1% of newborns will test positive with 99% of these outcomes being a false positive result, resulting in 21OHD screening to be one of the most cost ineffective neonatal screenings. These discrepancies can be cleared to a certain degree by having different threshold values for different gestational ages. In general, a better result is however obtained by performing a second round of tests on positive samples. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is used to detect 17OHP4 together with additional steroids (A4, cortisol, 11-deoxycortisol and DOF) and steroid ratios are used as test criteria. LC-MS/MS is however an expensive, low-throughput method and is therefore a costly procedure, especially if the high volume of positive test results is taken into account (Alonso-Fernández, 2016).

Misclassification based on hormonal profiles runs the risk of over- or under treatment during disease monitoring. Immunoassays are hampered by inaccuracies due to antibody specificity and variable interferences between individuals which can only be overcome by LC-MS/MS based technologies (Kamrath, Böhles, & Maser-Gluth, 2011). Screening of newborns for 17OHP4 levels is therefore highly controversial due to its poor diagnostic and economic efficiency. Studies employing gas chromatography-mass spectrometry (GC-MS) has identified urinary markers, which include pregnanetriolone, 5α-pregnan-3α, 17α-diol-20-one (Pdiol), pregnanetriol, and dihydropregnenolone that serve as more specific markers for 21OHD (Alonso-Fernández, 2016; Kamrath, Hartmann, Boettcher, Zimmer, & Wudy, 2016). GC-MS analyses of urinary samples is a more reliable diagnostic tool compared to immunoassay based techniques since it is non-invasive and can provide an overview of a whole spectrum of adrenal steroids in CAH patients, including glucocorticoid, androgen and 17OHP4 metabolites. In recent studies, the adrenal derived C11-oxy androgens were found as the dominant serum androgens in patients with classical CAH and urinary metabolites dominated by adrenal derived C11-oxy androgens allows the clinician to adequately access the overall androgen status in 21OHD patients (Kamrath, Wettstaedt, Boettcher, Hartmann, & Wudy, 2017). Similarly, DOF is regarded as a specific marker for 21OHD, enabling the clinician to distinguish between heterozygous carriers and NC 21OHD patients (Turcu et al., 2015). The presence of DOF demonstrates 11β-hydroxylation of 17OHP4, surpassing 21-hydroxylase metabolism due to the defective enzyme and diseases with elevated 17OHP4 levels from extra adrenal sources (eg. gonads, placenta), will not be coupled to DOF production (Christakoudi, Cowan, & Taylor, 2013; Kamrath et al.,

2011). For serum based studies DOE may also serve as a strong potential marker for 21OHD (Christakoudi, Cowan, & Taylor, 2013).

2.8 Treatment strategies

During 21OHD the loss of the negative glucocorticoid feedback on the hypothalamus is observed, causing increased corticotrophin and ACTH secretion which in turn leads to hyperplasia of the adrenal cortex and androgen excess (Mains, Lathi, Burney, & Dahan, 2007). Glucocorticoid treatments are therefore administered in an attempt to restore cortisol levels, suppress ACTH secretion and reduce adrenal androgen biosynthesis (Hardy & Cooper, 2010). Biochemical targets for the control of the disorder are ill defined and overtreatment can result in cortisol excess. A delicate balance between glucocorticoid and androgen levels therefore needs to be maintained. Therapy should aim at administering the lowest required dose while maintaining optimal control. Serum concentrations of 17OHP4, A4 and T are the most common indicators employed in monitoring glucocorticoid treatment (Kamrath et al., 2017) and when treatment is effectively monitored glucocorticoids should decrease the stimulation of the androgen pathway allowing normal growth and development. Anti-androgen treatment may however be useful as adjunctive therapy in adult females displaying hyperandrogenic signs despite strong adrenal suppression (Nimkarn et al., 2011).

2.9 Summary

In this chapter the central role which the adrenal plays during the production of steroid hormones has been highlighted. Adrenal steroidogenesis is crucial for numerous physiological processes, and even the smallest defect in any one of the enzymes within the adrenal steroidogenic pathways can have a cascade of detrimental effects with the clinical manifestation thereof prominent, and severe defects may even cause death. Since the outcome of adrenal steroidogenesis regulates various biological processes, adrenal diseases manifest in a broad spectrum of clinical symptoms. In 21OHD numerous clinical symptoms are observed and classified into three categories based on the severity of the enzyme defect. Adrenal androgen excess is however a hallmark of this disease and clinical symptoms can be traced back to specific androgens. Routine analysis of steroid levels for the classification of this disease is insufficient and alternative steroid metabolites are being investigated employing LC-MS/MS based technologies. As such, the metabolism by the downstream enzymes in the mineralocorticoid and glucocorticoid pathways, namely CYP11B and 11βHSD, are put forward as key enzymes, with the flux of accumulating products shunted towards these enzymes. In recent years C11-oxy steroids have enjoyed increasing interest and the production of C11-oxy C_{21} steroids by CYP11B and 11βHSD may contribute significantly towards the classification and identification of 21OHD. The metabolism of the accumulating products P4 and 17OHP4 by the adrenal-specific CYP11B isozymes catalysing the biosynthesis of 11OHP4 and DOF was therefore investigated and will be presented in chapter four.

CHAPTER 3

DOWNSTREAM PATHWAYS IN THE METABOLISM OF ADRENAL ANDROGEN PRECURSORS

3.1 Introduction

Androgen steroid hormones are vital for the development and maintenance of sex characteristics. The adrenal is an important source of androgen precursors and, to a lesser degree, androgens that can be secreted into circulation for further downstream metabolism. Metabolism in peripheral target tissue is involved in the biosynthesis of more potent androgens, as well as the inactivation of steroid hormones, thus maintaining physiological levels of active androgens. Enzyme expression within peripheral target tissue therefore plays a central role, dictating the ability of specific tissue to produce active androgens and inactivate steroid metabolites. Steroid levels in the target tissue microenvironment play a fundamental role in the progression of androgen dependent diseases and long term exposure has the ability to significantly influence the expression patterns of key enzymes. Changes in steroid production and metabolism are therefore often observed in clinical conditions. The hallmark of 21OHD presents as androgen excess with increased production of adrenal androgen precursors having a major impact on sex characteristics, specifically in female patients. In this chapter, the pathways of downstream metabolism in androgen target tissue will be reviewed.

3.2 Pathways involved in androgen metabolism

Humans, together with some primates, have the unique ability to produce large amount of androgen precursors in their adrenal. These adrenal androgen precursors are taken up in the bloodstream where they are circulated to peripheral target tissue. Once they have reach androgen responsive tissue, the expression of key enzymes may catalyse their conversion to more potent androgens that can bind to the AR to drive cell proliferation. The ability of a steroid metabolite to activate the AR refers to the potency of the compound, with DHT known as the most potent natural androgen. As such four pathways has been put forward that describes the conversion of adrenal androgen precursors to DHT (Penning, 2014; A. C. Swart & Storbeck, 2015). These pathways integrate key enzymes to catalyse the production of potent androgens and the activity of each pathway is therefore dependant on the expression of the relevant enzymes and the efficiency with which they catalyse available substrates. Since similar enzymes and substrates are involved in all of the metabolic pathways the dominant route followed in the production of DHT within a specific tissue are dependent on various factors including precursor availability, co-factor presence and the catalytic preference of enzymes towards different substrates. In 1988, the term intracrinology was coined, and describes the production of potent compounds which acts within the cell of origin, with secretion only

taking place once the compound has been inactivated. Recent studies have indicated that most peripheral tissue form part of the intracrine system, limiting the action of potent steroids to specific target tissue (Labrie et al., 2000; Labrie, 1991). This is in contrast to the adrenal that is part of the endocrine system, where the steroid metabolites produced are circulated in the bloodstream to exert their effect in neighbouring tissue.

In males, the testes are the principal source of androgens, specifically T, maintaining normal growth and development, with adrenal androgen precursors playing a secondary role. The role of the adrenal is however more prominent in females, and the production of androgen precursors and androgens in the adrenal is an important source that can have both physiological and pathological effects (F Labrie, Luu-The, Labrie, & Simard, 2001; Longcope, 1986). In androgen dependant disease states, such as 21OHD, the adrenal forms an integral part of androgen production. The main androgen precursors secreted by the adrenal is DHEA, A4 and 11OH4 (Rege et al., 2013). In addition to 11OHA4, significant levels of the C11-oxy C_{19} steroids, 11OHT, 11KA4 and 11KT has been detected in serum samples of 21OHD patients (Turcu et al., 2016). Even though not all of these metabolites exhibit androgenic activity, they contribute to the pool of circulating androgen precursors that may be metabolised to more potent androgens in peripheral target tissue, including prostate, skin and mammary glands.

3.2.1 The conventional C_{19} pathway

The testes is a common site for T production and upon reaching the prostate, this steroid will be converted to the more potent androgen, DHT by SRD5A. Both steroid 5α-reductase type 1 (SRD5A1) and SRD5A2 exhibit catalytic activity towards T, and this steroid therefore acts as a direct precursor in DHT biosynthesis. The production of DHT via this pathway was long thought to be the dominant route to DHT biosynthesis known as the conventional C_{19} pathway (Figure 3.1). The expression of both SRD5A isozymes has been reported in the prostate, with higher levels of SRD5A2 observed (Luu-The et al., 2008). In addition, the adrenal secretes low levels of T and also higher levels of androgen precursors DHEA and A4 (Rege et al., 2013), primarily formed in the adrenal zF. Therefore in addition to the limited T produced by the adrenal and high circulating T levels due to production of T in the testes, A4 and DHEA may also feed into this pathway. The first step involves the production of A4 from DHEA by 3βHSD2. The resulting A4 together with circulating A4 from the adrenal are converted to T by the action of 17βHSD enzymes, before the reduction to DHT by SRD5A isozymes. 17βHSD enzymes play a central role in the interconversion of weak and active androgens with 17βHSD3 and AKR1C3 (also known as 17βHSD5) the most widely accepted enzymes known to catalyse the conversion of A4 to T. 17βHSD3 is widely expressed in the testes (Luu-The, 2013), while AKR1C3 is reported to be the major subtype found in the prostate, with limited expression in the adrenal (Luu-The et al., 2008; Rege et al., 2013). The inactivation of potent steroids by 3αHSD and UDP-glucuronosyltransferase (UGT) enzymes will be discussed in *section 3.3*. T produced in the testes, is the major driving force behind the conventional C_{19} pathway with the adrenal androgen precursors making a secondary contribution.

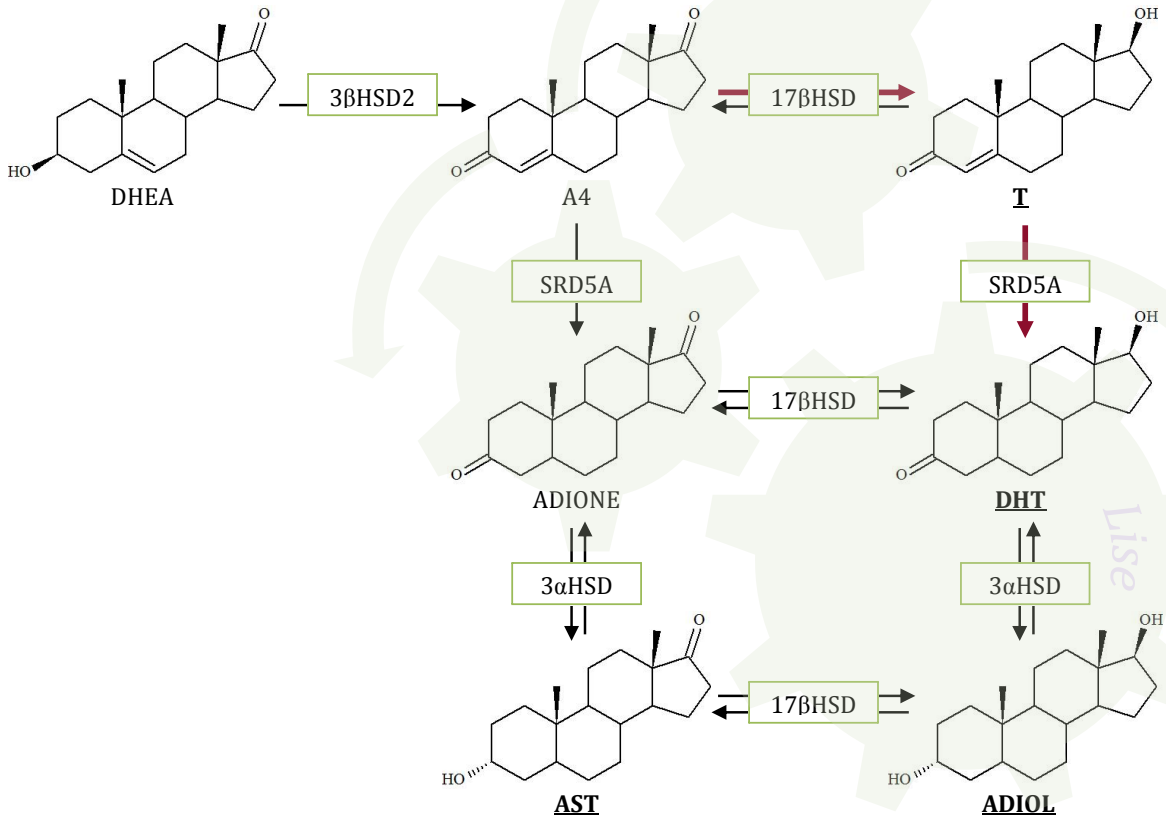


Figure 3.1 Conventional C_{19} pathway. Red arrows indicate pathway in which circulating T serves as a direct precursor for DHT production. Bold, underlined steroid metabolites are substrates for UGT2B enzymes.

3.2.2 The alternative 5α-dione pathway

In the last decade, accumulating evidence has led to the alternative 5α-dione pathway being reported as the major pathway involved in DHT production. This pathway, depicted in Figure 3.2, bypasses T, the conventional DHT precursor, with the adrenal androgen precursors DHEA and A4 having a primary role in the production of DHT. Both DHEA and A4 are substrates in this pathway and in this instance circulating A4 together with A4 produced via the reduction of DHEA by 3βHSD2, are first reduced to androstanedione (Adione) by the SRD5A isozymes before the conversion to DHT by 17βHSD enzymes. Studies have shown that this is the preferred route to DHT production in castration resistant prostate cancer (CRPC) cell models due to the substrate preference of SRD5A1 for A4 (Chang et al., 2011). The conversion of the adrenal androgen precursors therefore has a more prominent role in the production of potent androgens such as DHT than was first postulated, with this pathway now being accepted as the dominant route in DHT biosynthesis rather than the conventional C_{19} pathway where circulating T plays a central role. These findings are supported by studies that showed that SRD5A1 is upregulated in CRPC (Montgomery et al., 2008; Stanbrough et al., 2006; Titus et al., 2005), increasing the flux towards the production of Adione through the reduction of A4, before the conversion of Adione to DHT by 17βHSD enzymes. In CRPC, the adrenal androgen precursors are the main source of circulating precursors for potent androgen production due to chemical or physical castration preceding the progression towards CRPC. In the alternative 5α-dione

pathway reduction by the SRD5A enzymes is preferential to the reduction by the 17βHSD enzymes and the pathway will therefore follow the A4 → Adione → DHT route, bypassing T in the process (Chang et al., 2011). Similar to the products of the conventional C_{19} pathway, the products of the alternative 5α-dione pathway are substrates for 3αHSD and UGT2B enzymes that catalyse the inactivation of these steroids, leading to the subsequent secretion of potent steroid metabolites from peripheral target tissue, discussed in more detail in *section 3.3*.

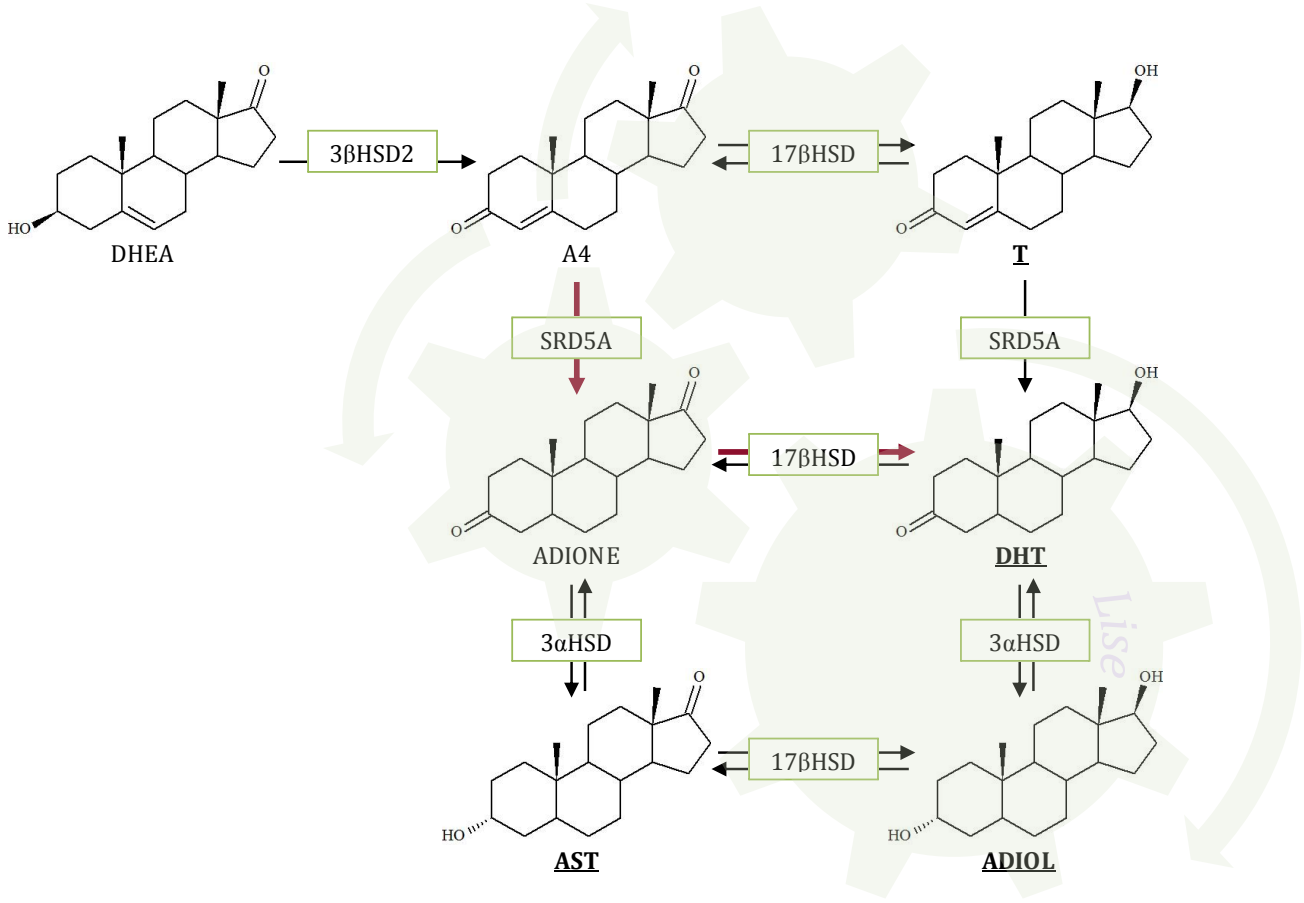


Figure 3.2 Alternative 5α-dione pathway. Red arrows indicate the pathway that bypasses T in DHT production. Bold and underlined steroid metabolites indicate substrates for conjugation by UGT2B enzymes.

3.2.3 The 11OHA4 pathway

In addition to the high levels of A4 and DHEA secreted by the adrenal, 11OHA4 is also a prominent androgen precursor, with the 11OHA4 levels in the adrenal vein samples of adults almost 2-fold that of A4 levels (Rege et al., 2013). The biosynthesis of 11OHA4 is catalysed by the adrenal CYP11B enzymes yielding the C11-hydroxyl product of A4 (Schloms & Swart, 2014; A. C. Swart et al., 2013) and was reported to be an inactivation step to prevent excessive androgen biosynthesis from the adrenal precursors. Studies in our laboratory have shown that 11OHA4 is a substrate for 17βHSD and SRD5A enzymes (A. C. Swart & Storbeck, 2015) and can be metabolised in peripheral target tissue in a similar manner as A4 in the conventional C_{19} and alternative 5α-dione pathways. The addition of a C11-oxy group adds a layer of complexity with the 11βHSD enzymes playing a prominent role catalysing the interconversion of C11-hydroxyl and keto groups (Bloem, Storbeck, Schloms, & Swart, 2013; Storbeck et al., 2013).

11OHA4, which exhibit limited androgenic activity, is now proven to be a precursor for more potent androgens, most prominently 11KDHT, in a manner similar to that in which DHT is produced from A4. In addition to 11OHA4, limited amounts of 11KA4, 11OHT and 11KT, biosynthesised in the adrenal, have been detected (Rege et al., 2013). The adrenal CYP11B isozymes therefore have the ability to produce 11OHT from T (A. C. Swart et al., 2013) and both 11OHA4 and 11OHT can be further metabolised to 11KDHT through the action of SRD5A, 11βHSD and 17βHSD (A. C. Swart & Storbeck, 2015) described in the 11OHA4 pathway (Figure 3.3).

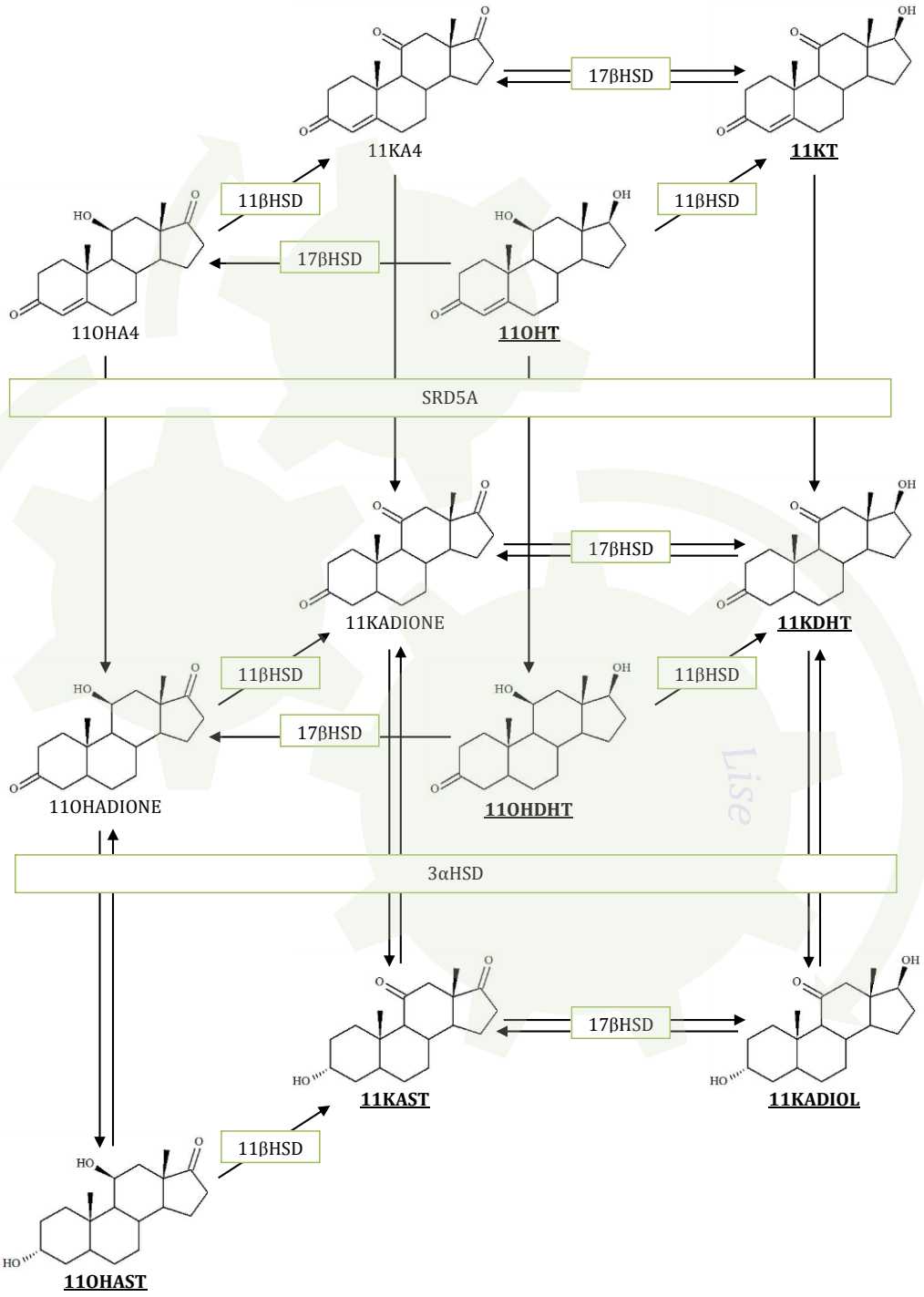


Figure 3.3 11OHA4 pathway. The conversion of the abundant adrenal steroid metabolite, 11OHA4, to more potent androgens. Bold underlined steroids can be conjugated by UGT2B enzymes.

In the adrenal the contribution of 11βHSD and 17βHSD enzymes are limited, with higher expression in peripheral tissue, resulting in the 11OHA4 pathway making a significant contribution in the production of potent androgens in androgen responsive tissue. Investigations into the catalytic activity of 17βHSD enzymes illustrated that the steroid metabolites with a C11-hydroxyl group are not preferred substrates for the reductive activity of the 17βHSD enzymes (Storbeck et al., 2013). 11OHA4 is therefore not converted to 11OHT by AKR1C3 or 17βHSD3, but rather first catalysed to 11KA4 by 11βHSD2, followed by the conversion to 11KT through the action of the 17βHSD enzymes. In addition, 11OHA4 acts as a substrate for SRD5A isozymes yielding 11β-hydroxyandrostanedione (11OHAdione) (Storbeck et al., 2013). Investigations furthermore showed that the oxidation reaction is the preferred direction for the conversion on C11, yielding mainly 11-keto steroids, and the metabolism of 11OHA4, 11OHAdione, 11β-hydroxyandrosterone (11OHAST), 11OHT and 11OHDHT are all substrates for 11βHSD2 yielding 11KA4, 11-ketoandrostanedione (11KAdione), 11-ketoandrosterone (11KAST), 11KT and 11KDHT respectively (Storbeck et al., 2013). Similar to the metabolism of 11OHA4, 11OHAdione is not a preferred substrate for AKR1C3 and will preferentially be converted to 11KAdione by 11βHSD2 before the reduction of C17 yielding 11KDHT. In addition, 11OHT, mainly produced in the adrenal from the 11β-hydroxylation of T (A. C. Swart et al., 2013), is converted to 11OHDHT through the action of the SRD5A isozymes before conversion by 11βHSD2, yielding 11KDHT (Storbeck et al., 2013; A. C. Swart & Storbeck, 2015). The steroids in the top tier of the pathway (Figure 3.3), 11OHA4, 11KA4, 11KT and 11OHT are substrates for the SRD5A enzymes, with SRD5A1 catalysing the conversion of A4 metabolites, 11OHA4 and 11KA4, more rapidly than that of 11OHT and 11KT (Storbeck et al., 2013). In the inactivation of the 11OHA4 pathway steroids, the C11-oxy steroids produced in the pathway yields substrates for 3αHSD and UGT2B enzymes as discussed in *section 3.3.*

The 11OHA4 pathway therefore contributes additional metabolites to the circulating androgen pool with the downstream metabolites being more androgenic than 11OHA4. Conversion of 11KA4 and 11KAdione by 17βHSD enzymes increases the agonist potential of these metabolites, with the subsequent SRD5A reaction further increasing the potency of 11OHT and 11KT, yielding 11OHDHT and 11KDHT. Of significance however, is the production of 11KT and 11KDHT, which have been shown to have AR potencies comparable to that of T and DHT respectively (Bloem et al., 2015). 11KDHT is therefore a full AR agonist at 1 nM, highlighting the importance of this pathway in androgen responsive tissue. In 21OHD patients increased levels of 11OHA4, 11KT, 11KA4 and 11OHT was observed in adrenal vein samples, showing the major contribution that the adrenal has in the production of potent androgens (Turcu et al., 2015).

3.2.4 The backdoor pathway

In addition to the pathways above, an additional pathway leading to DHT production has been reported (Locke et al., 2008). In this pathway the conventional adrenal metabolites DHEA, A4 and T are bypassed, and DHT production occurs through the metabolism of the adrenal steroids P4 and 17OHP4. This pathway is therefore of specific interest in cases of 17OHP4 accumulation as is observed in the case of 21OHD.

Similar to pathways discussed in previous sections, SRD5A and 17βHSD are involved in the metabolism of P4 and 17OHP4, however CYP17A1 and 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2) are prominent participants in the formation of DHT. The pathway is activated once P4 or 17OHP4 is reduced by SRD5A, yielding dihydroprogesterone (DHP) and 5α-pregnan-17α-ol-3, 20-dione (Pdione) respectively. These steroids are substrates for AKR1C2 and are converted to their respective 3α-, 5α- reduced C_{21} steroid products, 5α-pregnan-3α-ol-20-one (AlloP5) and Pdiol. The resulting steroids are catalysed by CYP17A1 to produce androsterone (AST), which in turn can be metabolised by 17βHSD3 and 17βHSD6 to yield DHT with Adiol as intermediate. The production of DHT from reduced C_{21} steroids, bypassing the conventional DHT precursors is known as the backdoor pathway (Figure 3.4).

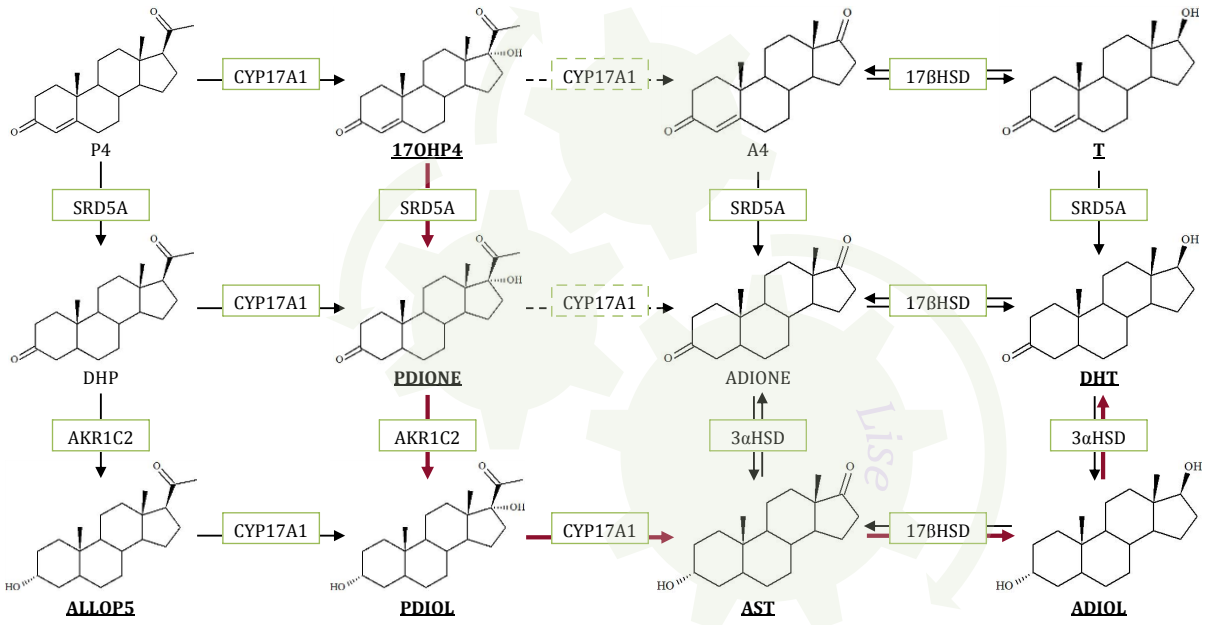


Figure 3.4 Backdoor pathway (red arrows) indicates the production of DHT proceeding via the metabolism of 17OHP4, bypassing the conventional adrenal androgen precursors. Underlined and bold steroid metabolites are potential substrates for UGT2B enzymes.

As discussed in the previous chapter, the 17, 20-lyase activity of CYP17A1 is a prerequisite for the biosynthesis of C_{19} steroid metabolites. Since CYP17A1 exhibits limited lyase activity towards 17OHP4, even in the presence of cytb5 (Lee-Robichaud et al., 1995), the backdoor pathway provides an alternative for the metabolism of 17OHP4. In the backdoor pathway the C4-5 double bond of the C_{21} steroid, 17OHP4, is firstly reduced, followed by the 3α-reduction of the C3 hydroxyl group before the conversion by CYP17A1 to yield the C_{19} steroid, AST. These reductions by SRD5A and AKR1C2 appear to increase the affinity of the CYP17A1 lyase activity for the substrate, even in the absence of cytb5, and the resulting Pdiol is to date reported to be the best substrate for the lyase activity of CYP17A1 (Gupta, Guryev, & Auchus, 2003). In cases of 17OHP4 accumulation, where minimal A4 is produce by the CYP17A1 lyase activity (Lee-Robichaud et al., 1995), this steroid may be utilised by SRD5A, activating the backdoor pathway. The reduction by the SRD5A isozymes is therefore the initial, activating step in this pathway and

studies has reported that 17OHP4 is the preferred substrate for 5α-reductase activity over A4 or T (Frederiksen & Wilson, 1971). The 5α-reduced substrates DHP and Pdione are however, not suitable substrates for the lyase activity of CYP17A1 (Gupta et al., 2003) and are instead rather metabolised by AKR1C2 (Flück et al., 2011). DHP is rapidly converted to Pdione, with limited amounts of the 17, 20-lyase product, Adione being formed, even after the addition of cytb5 (Gupta et al., 2003). Metabolism of DHP by CYP17A1 is therefore restricted to the 17α-hydroxylation, and Pdione is a weak substrate for the lyase reaction. In contrast with this, AlloP5 and Pdiol, the resulting steroid metabolites of the reduction catalysed by AKR1C2, are rapidly metabolised by CYP17A1 yielding AST (Gupta et al., 2003). Studies regarding the affinity of different substrates showed that 3α, 5α-reduced metabolites are excellent substrates for human CYP17A1 and both AlloP5 and Pdiol are metabolised more rapidly than P4 and P5 (Gupta et al., 2003). Similarly, reports have indicated that C3 and C5 of the steroid structure, the functional C3 moiety and the presence or absence of the C4-5 double bond are key determinants in steroid metabolism (Christakoudi, Cowan, Christakudis, et al., 2013). The reduction of the C3 keto group can therefore perhaps be regarded as an activation step in the metabolism of C_{21} steroids in this pathway, presenting substrates that are effectively lyased by CYP17A1 to produce C_{19} steroids, precursors for potent androgens.

The expression of both CYP17A1 and SRD5A is essential in the backdoor pathway, and their affinity for the presented substrates will influence the flux through this pathway, therefore having a major influence on androgen levels. SRD5A is regarded as the gatekeeper of the backdoor pathway, since the production of the key C_{21} intermediate, Pdiol, is dependent on this enzyme. Once this intermediate is formed the production of AST can take place modulated by the 17, 20-lyase activity of CYP17A1 (Kamrath, Hartmann, Remer, & Wudy, 2012). The SRD5A isozymes play a prominent role in the production of active androgens and are widely expressed in androgen responsive tissue such as the prostate and skin (Luu-The et al., 2008; Martel, Melner, Gagné, Simard, & Labrie, 1994). P4 and 17OHP4 are excellent substrates for both SRD5A enzymes (Frederiksen & Wilson, 1971). As discussed in the previous chapter, the expression of CYP17A1 is essential for the production of androgens since the 17, 20-lyase activity of this enzyme catalyse the conversion of C_{21} steroids towards C_{19} steroids. CYP17A1 is mainly expressed in the adrenals and steroidogenic tissue and is regulated by electron transport of NADH from POR. Limited activity towards 17OHP4 is observed and following the cleavage of 17OHP5 to DHEA, the Δ^5-pathway is the principle route in the production of C_{19} steroids (Auchus et al., 1998). Conversion of 17OHP4 to A4 is rarely observed, even in cases of abnormally high 17OHP4 levels, as is the case in 21OHD. CYP17A1 therefore has a modulatory role, and will determine the flux through the Δ^4-, Δ^5- or the backdoor pathway. With Pdiol being the optimal substrate for the lyase activity of CYP17A1 and, in contrast to the classical pathways, CYP17A1 catalyses the conversion of Pdiol to AST even in the absence of cytb5, while the addition of cytb5 increases the reaction efficiency significantly (3-fold) (Gupta et al., 2003). Given CYP17A1's affinity towards 17OHP4, 17OHP5 and Pdiol, together with the expression of cytb5 these factors will determine substrate conversion in 21OHD, dictating the flux through the pathways and ultimately androgen production (Kamrath, Hartmann, et al., 2012). The high affinity of SRD5A towards 17OHP4, together with

the negligible lyase activity of CYP17A1 towards this substrate, therefore promotes the backdoor pathway to produce androgens.

3.3 Androgen inactivation

The expression of the AR together with active androgens is crucial for the translation of active androgens into physiological effects. As such, the inactivation of steroid hormones is important to prevent adverse steroid influences in terminating the androgen signal. The inactivation of steroid hormones is an additional physiological tool implemented to fine-tune hormonal balance and regulation. In peripheral target tissue two enzymes have a central role in the inactivation of potent androgens, namely AKR1C2 and UGT2B.

3.3.1 AKR1C2

The 3α-hydroxysteroid dehydrogenase type 3 (3αHSD3) enzyme has been identified as an aldo-keto reductase (AKR) enzyme that belongs to the AKR1C family, and preferentially exhibits reductive catalytic activity (Rizner et al., 2003). This enzyme, also known as AKR1C2, is prevalent in peripheral tissue including the prostate, skin and brain (Luu-The et al., 2008; Penning et al., 2000). AKR1C2 catalyses the reduction of the C3 keto group, yielding steroids with C3 hydroxyl groups. In the conventional C_{19}, alternative 5α-dione and 11OHA4 pathways, the 3α-reduction yields steroid products less potent than their substrates and therefore elicits a weaker androgenic activity. Adione and DHT are both substrates for AKR1C2 yielding AST and Adiol respectively (Bauman, Steckelbroeck, Williams, Peehl, & Penning, 2006; Rizner et al., 2003). In addition, unpublished data from our laboratory has shown that in the 11OHA4 pathway 11OHAdione, 11KAdione and 11KDHT were catalysed by AKR1C2 yielding the inactive steroid metabolites 11OHAST, 11KAST and 11-ketoandrostanediol (11KAdiol). The 11OHDHT product, 11β-hydroxyandrostanediol (11OHAdiol), has to date, not been identified. Importantly, AKR1C2 has been shown to be incapable of the reverse reaction catalysing AST to DHT (Ji, Chang, van den Berg, Stanczyk, & Stolz, 2003; Rizner et al., 2003), strongly supporting its preferred reductive activity. In addition to yielding weaker androgens, AKR1C2 produces steroids with a C3 hydroxyl group and as such contributes to the pool of steroids presenting substrates for conjugation by the UGT enzymes. The AKR1C2 enzyme therefore prepares steroid metabolites for secretion yielding potential substrates for UGT2B enzymes. Therefore A4 metabolites, in addition to T (that contains a C17 hydroxyl group) can subsequently be inactivated. It is important to note that even though AKR1C2 aids in the secretion of AR ligands and ligand precursors within the conventional C_{19}, alternative 5α-dione and 11OHA4 pathways, in the backdoor pathway the reduction catalysed by this enzyme results in substrates that are readily acted on by the CYP17A1 lyase activity. Therefore, even though these steroids may potentially be glucuronidated by the UGT enzymes, significant conversion by CYP17A1 is detected.

Of similar importance is the role of the human oxidative 3αHSD (RL-HSD), also known as 17βHSD6, that is reported to catalyse the reverse reaction of that catalysed by AKR1C2. 17βHSD6 catalyses the

conversion of Adiol to DHT and AST to Adione, albeit to a lesser degree, and has been identified as the main enzyme catalysing these conversions. 17βHSD6 expression has been reported to be 15 times higher than other candidate enzymes in the prostate, exhibiting only oxidative activity (Bauman et al., 2006; Miller & Auchus, 2011; Mohler et al., 2011; Penning, Bauman, Jin, & Rizner, 2007). Studies carried out in prostate cancer cells further revealed increased DHT production correlates with increased 17βHSD6 mRNA and protein expression. Furthermore the addition of Adiol to prostate cells elicits an AR transactivation response in parallel with increased DHT biosynthesis (Mohler et al., 2011). These findings are supported by studies into the co-localisation of 17βHSD6 and the AR (Bauman et al., 2006). *In vivo* studies in mice also showed that administration of Adiol resulted in a 28-fold and 4.3-fold increase in DHT and AST levels respectively (Mohler et al., 2011). The reactivation of Adiol to form DHT is therefore a possible mechanism sustaining AR transactivation in androgen deprived environments. This is especially evident in prostate cancer patients treated with ADT and studies in LNCaP cells in which the expression of 17βHSD6 increased with the number of passages (Ishizaki et al., 2013). Adiol itself does not bind the AR but conversion of this steroid metabolite by 17βHSD6 yielding DHT can drive cell proliferation in the prostate (Bauman et al., 2006). The role of 17βHSD6 is of unequivocal importance in the backdoor pathway. The conversion of Adiol to DHT by 17βHSD6 is the last step in this pathway and a prerequisite for the activation of the AR. The interplay between AKR1C2 and 17βHSD6 in the interconversion of Adiol and DHT is further complicated by the expression of UGT2B enzymes that irreversibly inactivates these steroid metabolites (*section 3.3.2*). Studies investigating the catalytic activity of 17βHSD6 towards C11-oxy steroid metabolites in the 11OHA4 pathway are current underway in our laboratory. In the case of Adiol production bypassing DHT, the accumulation of Adiol may direct the flux to DHT production which in turn will bind to the AR and elicit physiological responses. Both AKR1C2 and 17βHSD6 therefore have activation roles in the backdoor pathway, crucial in the regulation of AR transactivation and may therefore have an important role in androgen biosynthesis and metabolism in pathological states such as 21OHD.

3.3.2 UGT2B enzymes

In humans three different UGT enzymes, UGT2B7, UGT2B15 and UGT2B17, have been identified that can conjugate androgen metabolites in the prostate. The UGT enzymes are widely expressed throughout the body, mainly within androgen responsive tissue such as the prostate, skin and mammary glands (Turgeon, Carrier, Lévesque, Hum, & Bélanger, 2001). These enzymes facilitated the transfer of a glucuronic acid group from uridine diphosphoglucuronic acid (UDP) to steroid metabolites with C3 and C17 hydroxyl groups. This transfer is irreversible and the addition of a glucuronide moiety on the steroid molecule inhibits reactivation and the potential interaction with the AR, thereby preventing the transactivation of androgen responsive genes. Furthermore, the glucuronidation of steroid metabolites increases the polarity of the compound, facilitating in the secretion thereof. UGT enzymes therefore play a crucial role in the inactivation and secretion of C_{19} steroid metabolites. Both AKR1C2 (as discussed in *section 3.3.1*) and 17βHSD enzymes contribute to the production of steroid metabolites containing a hydroxyl group on C3 or C17 respectively, thereby augmenting the UGT activity through the production of possible substrates for

these enzymes. DHT, that only contains a C17 hydroxyl group, was identified as a weak substrate for UGT2B7, which will more readily conjugate AST on the available C3. UGT2B7 exhibited the highest activity towards Adiol (on C3), with Adiol not observed as a substrate for glucuronidation at C17 by this enzyme (Bélanger, Pelletier, Labrie, Barbier, & Chouinard, 2003). UGT2B7 therefore mainly exerts glucuronidation activity towards C3-hydroxyl groups. On the other hand, UGT2B15 is specific to C17-hydroxyl groups and will preferably glucuronidate Adiol, followed by DHT and T, yielding Adiol-glucuronide (Adiol-G), DHT-glucuronide (DHT-G) and T-glucuronide (T-G) respectively. UGT2B17 has the ability to glucuronidate steroids on both the C3 and C17 position. This enzyme glucuronidates Adiol with similar efficiency than the previously mentioned enzymes, but shows higher efficiency with AST and DHT compared to UGT2B7 and UGT2B15. Adiol (that can receive a glucuronic acid group on C3 or C17) is observed as the best substrate, followed by DHT, T and lastly AST. Adiol-G is the most abundant glucuronidated steroid observed in plasma for both males and females (Bélanger et al., 2003; Turgeon et al., 2001). Studies investigating the metabolism of the C11-oxy steroids by UGT2B enzymes are currently conducted within our labarotory. Glucuronidation of 11KDHT has been reported, however, the conjugation of 11KT is not as efficient, with significantly lower levels of glucuronidation towards 11KT being observed (du Toit et al., 2017).

3.4 Summary

In this chapter the downstream metabolism of adrenal androgen precursors and the production of active androgens have been presented. It is clear that adrenal androgen precursors have a major role in androgen physiology, contributing to the pool of active androgens. During 21OHD, not only is there an increased flux towards the biosynthesis of adrenal androgen precursors, but there are also significant levels of the CYP21A2 substrates, P4 and 17OHP4. Since these steroids are secreted into circulation, and with the metabolism by the enzymes described in the backdoor pathway, these steroids may contribute significantly to the production of potent androgens. In chapter 5 the metabolism of 17OHP4, together with the C11-oxy forms of this steroid, DOF and DOE, by the enzymes of the backdoor pathway will be investigated.

CHAPTER 4

THE BIOSYNTHESIS OF C11-OXY C_{21} STEROIDS BY ADRENAL CYP11B1 AND CYP11B2 ENZYMES

Adrenal steroidogenesis plays a central role in the production of steroid hormones and adrenal disorders have detrimental effects on normal physiological homeostasis. 21OHD is the most prevalent inborn metabolic disorder, presenting as an enzyme defect in CYP21A2, the enzyme catalysing the biosynthesis of the mineralocorticoid and glucocorticoid precursor steroids in the adrenal. In 21OHD, the flux towards these pathways is diminished, due to limited conversion of P4 to DOC and 17OHP4 to 11-deoxycortisol. This leads to accumulation of CYP21A2 steroid substrates, P4 and 17OHP4 and as such, the metabolism of these steroids may commence through the downstream adrenal steroidogenic enzymes. In adrenal steroidogenesis, the CYP11B isozymes catalyse the production of active mineralocorticoids (ALDO) and glucocorticoids (cortisol), from DOC and 11-deoxycortisol. Our research group has recently demonstrated that the 11β-hydroxylation of A4, an adrenal androgen precursor, produced 11OHA4 by the catalytic conversion of CYP11B1 and CYP11B2 (A. C. Swart et al., 2013). Studies have identified 11OHP4 and DOF in serum samples of 21OHD patients, indicative of the 11β-hydroxylation of P4 and 17OHP4 (Turcu et al., 2015). *In vitro* studies investigating the metabolism of P4 and 17OHP4 through the catalytic conversion of the CYP11B isozymes has however not been assayed. The biosynthesis of C11-oxy C_{21} steroids may contribute significantly to the circulating pool of adrenal metabolites, serving as substrates for more potent steroid hormones in peripheral target tissue.

The biosynthesis of C11-oxy steroids in the adrenal present potential substrates for the 11βHSD enzymes. The role of the 11βHSD activity towards cortisol and cortisone are well-defined. 11βHSD2 catalyses the inactivation of cortisol, converting the C11 hydroxyl group to a keto group, forming inactive cortisone, which in doing so has a regulatory role regarding glucocorticoid levels and mineralocorticoid receptor (MR) activation. Conversely, 11βHSD1 catalyses both oxidative and reductive reactions dependant on the co-factor levels available and is involved in the regeneration of active cortisol from cortisone by catalysing the conversion of the C11-keto group to a hydroxyl group (Stewart, 2003). Our group recently established the 11OHA4 pathway that describes the formation of potent active androgens in the downstream metabolism of the abundant inactive adrenal androgen precursor, 11OHA4. In this pathway, 11βHSD enzymes play a central role and we have shown that C11-oxy C_{19} steroids are more potent androgens when in their keto form. These findings questioned the inactivation regulatory function assigned to 11βHSD2, considering this enzyme catalysed the respective conversion of 11OHA4 and 11OHT to 11KA4 and the more potent 11KT (A. C. Swart et al., 2013; A. C. Swart & Storbeck, 2015). It is therefore prudent to surmise that the biosynthesis of the C11-oxy C_{21} steroids, 11OHP4 and DOF, in the adrenal may lead to

11KP4 and DOE (Figure 4.1) through the action of the 11βHSD isozymes, and also contributing more potent downstream steroid hormones.

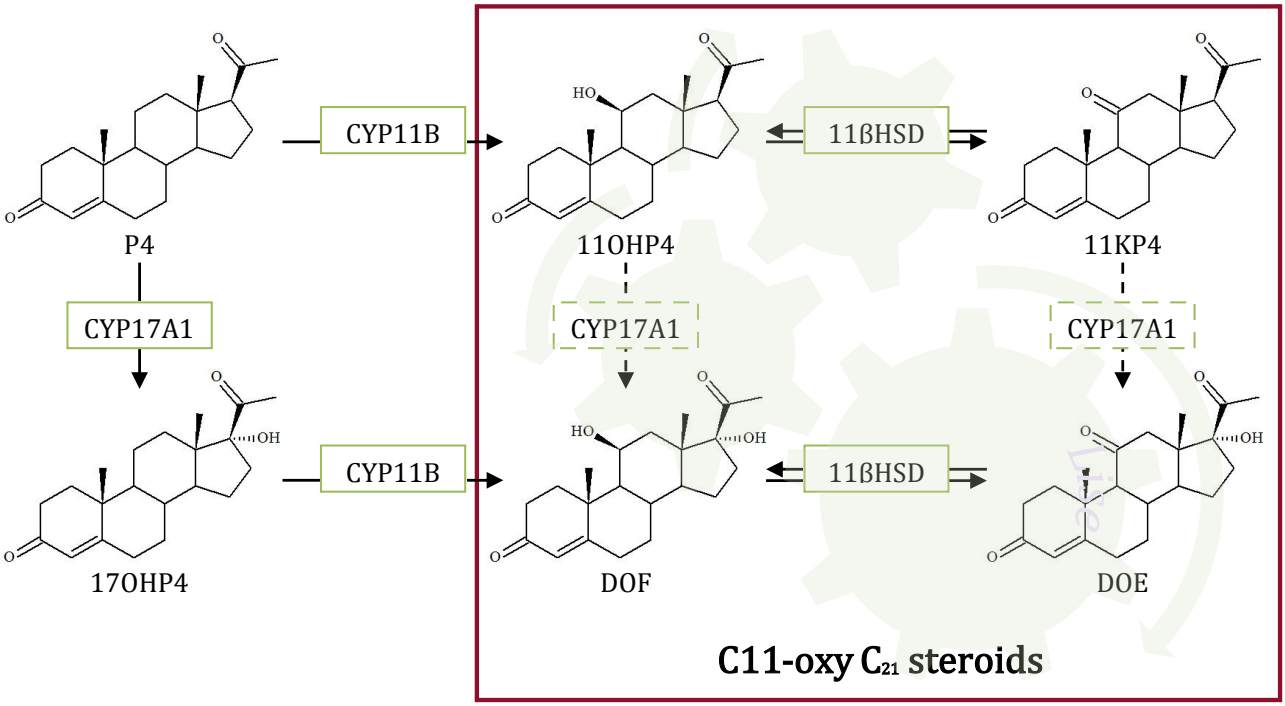


Figure 4.1 Biosynthesis of C11-oxy C21 steroids in the 21OHD adrenal due to increased P4 and 17OHP4 concentrations. Enzymes conversions indicated in dashed boxes have not yet been confirmed.

Although DOF was identified in 21OHD patients samples over 50 years ago, interest into this molecule was overshadowed by investigations into 17OHP4 and cortisol (Milewicz et al., 1984; Nahoul, Adeline, & Bercovici, 1989; Wieland, Maynard, Riley, & Hamwi, 1965). In recent years, interest in the adrenal CYP11B isozymes has increased with the discovery and development of the 11OHA4 pathway (Bloem et al., 2013; Storbeck et al., 2013; A. C. Swart et al., 2013; A. C. Swart & Storbeck, 2015). Furthermore, the advances made in LC-MS/MS based technologies has made the accurate and sensitive analyses of samples possible (Bloem et al., 2015). In the milieu of 21OHD, both 11OHP4 and DOF may play a central role in the disease state and clinical studies investigating 21OHD are including these steroids in hormone profiles of patients (Turcu et al., 2015). Further investigations of both 11OHP4 and DOF are required, and the accurate identification and quantification of these steroids are now possible using LC-MS/MS based methods.

The aim of this investigation was to determine the production of 11OHP4 and DOF and therefore the catalytic activity of the CYP11B isozymes towards P4 and 17OHP4 over the course of 24 hours were determined in the transiently transfected HEK293 cell model system.

4.1 Methodology

4.1.1 Materials

Steroids, 11OHP4, 11OHA4, 17OHP4 and DOF were purchased from Steraloids (Wilton, USA), while A4 and P4, methyl tertiary-butyl ether (MTBE) and Dulbecco's modified Eagle's Medium (DMEM) was bought from Sigma-Aldrich (St. Louis, MO, USA). Wizard® Plus Midipreps DNA purification system was supplied by Promega Biotech (Madison, WI, USA) and the Corning® CellBIND® Surface 24-well plates was obtained from Corning® Life Sciences (Corning, NY, USA). XtremeGene HP® DNA transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). Penicillin-streptomycin, fetal bovine serum (FBS) and trypsin-EDTA were supplied by Gibco BRL (Gaithersburg, MD, USA). The deuterated steroids D2-T (Testosterone 1,2-D2, 98%) and D9-P4 (Progesterone 2,2,4,6,6,17A,21,21,21-D9, 98%) were obtained from Cambridge isotopes (Andover, MA, USA). Trypan blue stain (0.4%) together with cell count plates were acquired from Invitrogen (Eugene, USA). All other chemical reagents were of the finest quality and supplied by trustworthy scientific distributers.

4.1.1.1 Cell lines and vectors

HEK293 cells were purchase from American Type Culture Collection (Manassas, VA, USA). HEK293 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were kept in a stable environment at 37 °C, 5% CO_2 and 90% relative humidity. Before each experiment cells were removed from cryogenic storage and cultured for at least three passages before being used in experiments. Confluent cells were replated at a cell concentration of $1x10^5$ cells/ml for normal growth maintenance. Cells were tested for mycoplasma contamination on the third passage cycle. The CYP11B1 and CYP11B2 vector constructs were kind gifts from Prof Walter Miller and the ADX (adrenal ferredoxin) vector construct was donated by Prof RC Tuckey.

4.1.2 Extraction of plasmid DNA

DNA (human) from mammalian expression vectors, pCMV/CYP11B1, pCMV/CYP11B2, pCIneo/ADX and the control plasmid pCIneo, was prepared by streaking out frozen *E.coli* stocks (JM109 strain) on ampicillin (100 µg/mL) LB-agar plates and incubated overnight at 37 °C. Single colonies were selected and inoculated in 5 mL LB media, containing 5 µL ampicillin (100 mg/mL) and thoroughly mixed. Starter cultures were incubated at 37 °C for 8 hours. After the first incubation, a second larger inoculate was made by adding 150 µL of the starter culture to 150 mL LB media and 150 µL Ampicillin (100 mg/mL). Cultures were incubated for a further ±16 hours at 37 °C on a Innova shaking-incubater (New Brunswick, Canada) at 225 rpm. Bacterial cells were lysed and plasmids purified with a DNA extraction column using the Wizard® *Plus* Midipreps DNA Purification System in accordance with the manufacturer's instructions. DNA plasmid concentrations were calculated using UV spectrophotometry at 260/280nm on the Cary60 UV-Vis (Agilent technologies; Santa Clara, CA, USA). Purified DNA was stored at -20°C until needed.

4.1.3 Enzyme assays in transiently transfected HEK293 cells

Cells were grown in DMEM culture media supplemented with 10% FBS and 1% penicillin-streptomycin. The environment was kept stable at 37 °C, 5% CO_2 and 90% relative humidity. Confluent cells were seeded in 24 well Corning® CellBIND® surface plates at a cell density of $2x10^5$ cells/mL. After a 24 hour incubation period, cells were transiently transfected with 0.5 µg plasmid DNA per well using XtremeGene DNA transfection reagent in accordance with the manufacturer's instructions. Cells were co-transfected with pCMV/CYP11B1 or pCMV/CYP11B2 together with pCIneo/ADX plasmid DNA as the cell models does not optimally support the redox function of the enzymes. The pCIneo plasmid containing no DNA insert was used as a negative control. Cells were incubated for 72 hours before the media was aspirated and replaced with fresh media containing 1 µM of the appropriate steroid (P4, 17OHP4 or A4). Over the course of 24 hours, aliquots were collected in triplicate at specific time intervals, placed in screw cap tubes and stored at 4 °C. Steroids were extracted at room temperature by adding 100 µL internal standard (15 ng of D2-T and D9-P4) and 3 mL MTBE to each sample (1:3 steroid extraction ratio of media to organic solvent). Samples were vortexed for 10 minutes and place at –20 °C for 1 hour. Organic phase were transferred to labelled test tubes, placed at 45 °C and dried under a stream of nitrogen. The resulting dried samples were resuspended in 150 µL 50% MeOH and stored at –20 °C until UHPLC-MS/MS analyses. The aliquot of each time interval of P4 and 17OHP4 were chromatographed together as a single sample (multiplex analysis) since substrates and products could be analysed without interference by a specific multiple reaction monitoring (MRM) in four different channels separating these steroid metabolites effectively.

4.1.4 LC-MS/MS analyses of steroids

4.1.4.1 Accurate mass determination of 11β-hydroxylated steroids

The purity of the 11OHP4 and DOF standards were firstly analysed and the accurate mass determined prior to assaying the metabolism of P4 and 17OHP4 using the Waters Synapt G2 quadropole time-of-flight mass spectrometer (Q-tof MS) (Waters Corporation, Milford, MA, USA) in positive electrospray ionisation (ESI^+) mode and MRM mode. An ACQUITY UHPLC (Waters Corporation, Milford, MA, USA) was used as interface with a BEH C_{18} column (2.1 x 100 mm) (Waters Corporation, Milford, MA, USA) for chromatographic separation of the steroids. The total ion count (TIC) of parent molecules ranging from 50-1200 Da was scanned. Leucine enkephalin was used as reference compound with a lock mass of 556.2771 Da. The lock spray parameters were set to a reference cone voltage of 15 V and reference capillary voltage of 3 kV. The instrument was calibrated with sodium formate and instrumental parameters as follow: capillary voltage, 2.5 kV; cone voltage, 15V; source temperature, 120 °C; desolvation temperature, 275 °C; cone gas flow 50 L/hour, desolvation gas flow, 650 L/hour, lock spray infusion flow rate, 1 µL/minute; collision energy, 4 eV; column temperature, 40 °C and acquisition mass range of 150-600 *m/z*. An injection volume of 5 µL was used and a total run time of 7 minutes was allowed using 1% formic acid (A1) and acetonitrile (B1) as solvents with a flow rate of 0.4 mL/minute. Initially a 50%

A:B solvent ratio was used, following a linear gradient to reach 100% of solvent B at 4 minutes, kept for 1 minute, before returning to a 50:50 ratio by 5.1 minutes, maintained until the end of the run. Water was used as weak solvent wash (1000 µL) and acetonitrile as strong solvent wash (500 µL). Data was collected and analysed using the MassLynx 4.1 software package.

4.1.4.2 Separation and quantification of steroid metabolites using UHPLC-MS/MS

Steroid metabolites were separated and analysed using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Standard steroids stocks of 2 mg/mL were prepared for P4, 11OHP4, 17OHP4, DOF, A4 and 11OHA4 using absolute ethanol. A standard dilution series was prepared in experimental DMEM for a standard range of 0.2–2 µg/mL. For each standard a 500 µL aliquot was prepared and steroids extracted as describe in *section 4.1.3*. Steroid metabolites were separated on an ACQUITY UHPLC (Waters Corporation, Milford, MA, USA) using a Kinetex PFP column (2.1 mm x 100 mm x 2.6 µm) (Phenomenex Corporation, Torrance, CA, USA) coupled with a Xevo triple quadropole mass spectrometer (QTMS) (Waters Corporation, Milford, MA, USA). ESI^+ mode and MRM mode throughout, scanning for a mass range of 40-2040 Da, acquiring data from 150-600 *m/z*. Instrumental settings as follow: capillary voltage, 3.5 kV; source temperature, 140 °C; desolvation temperature, 400 °C and desolvation gas flow, 800 L/hour, cone voltage, 15-53.35 V and cone gas flow, 50 L/hour; collision gas flow, 0.2 mL/minute; MS mode collision energy, 4 eV and MS/MS mode collision energy, 20 eV; column temperature, 40 °C. Samples (5 µL) were chromatographed for a total of 5 minutes using a mixture of methanol:acetonitrile:isopropanol (49:49:2) as solvent A1 and 1% formic acid in deionised water (B2) for the mobile phase at a flow rate of 0.4 mL/minute using the solvent gradient program depicted in Table 4.1.

Table 4.1 UHPLC solvent parameters for the chromatographic separation.

	Time (minutes)	Flow rate (mL/minute)	Solvent A (%)	Solvent B (%)	Curve gradient
1	Initial	0.400	85	15	0
2	0.50	0.400	55	45	6
3	3.50	0.400	35	65	6
4	3.60	0.400	0	100	6
5	4.00	0.400	0	100	6
6	4.01	0.400	85	15	6
7	5.00	0.400	85	15	6

Data was collected and analysed using the MassLynx 4.1 Software package.

4.1.5 Statistical analyses

GraphPad Prism 5 was used for all statistical analyses. Experiments were carried out in triplicate and the results displayed as the mean value ±SEM. Statistical significance was investigated using an unpaired t-test or one-way ANOVA with a Dunnett's post-test. P-values lower than 0.05 were taken as a significant difference.

4.2 Results

Although the production of DOF, and more recently 11OHP4, has been attributed to the 11β-hydroxylation of P4 and 17OHP4 by CYP11B isozymes, the catalytic activity of the CYP11B isozymes towards P4 and 17OHP4 have not been described or reported to date.

4.2.1 Plasmid DNA analyses

To investigate the catalytic conversion of the CYP11B isozymes, plasmid DNA vectors containing the genes of interest were prepared and purified. Table 4.2 summarises the average yield obtained per 100 mL culture together with the 260/280 ratio indicating the purity of the vector construct. A ratio of 1.8-2 is desirable. A 260/280 ratio higher than 2 is indicative of RNA contamination, while values below 1.8 points towards protein contamination. Isolated plasmids were used for enzyme assays in transfected cell systems.

Table 4.2 Plasmid yield and purity.

cDNA	Yield (µg)	Ratio
pCMV/hCYP11B1	482.1	1.87
pCMV/hCYP11B2	346.2	1.82
pCIneo/hADX	346.8	1.94
pCIneo	555.6	1.85

4.2.2 LC-MS/MS steroid analysis of the C11-oxy C_{21} steroids

LC-MS/MS analyses of 11OHP4 and DOF indicated that there were no steroid contaminants when standards were scanned between 50-1200 Da with only the relevant steroid present when scanning the acquisition mass range of 150-600 *m/z*.

4.2.2.1 Accurate mass-determination of 11OHP4 and DOF

Once the accurate mass of each peak representing 11OHP4 and DOF (Figure 4.2) was determined, further fragmentation using Q-tof-MS/MS was carried out. The precursor ions where selected at their specific $[M+H]^+$ mass during the first MS run and further fragmented in the coupled collision cell before the second

round of MS, displaying the fragmentation of the selected steroid metabolite. A broad spectrum of product ions were obtained and selection of specific ions for the MRM set-up on the UHPLC-MS/MS is based on signal intensity and fragment specificity, since steroid structures are closely related and may share specific $[M+H]^+$ masses. Accurate mass determinations of 11OHP4 and DOF are summarised in Table 4.3.

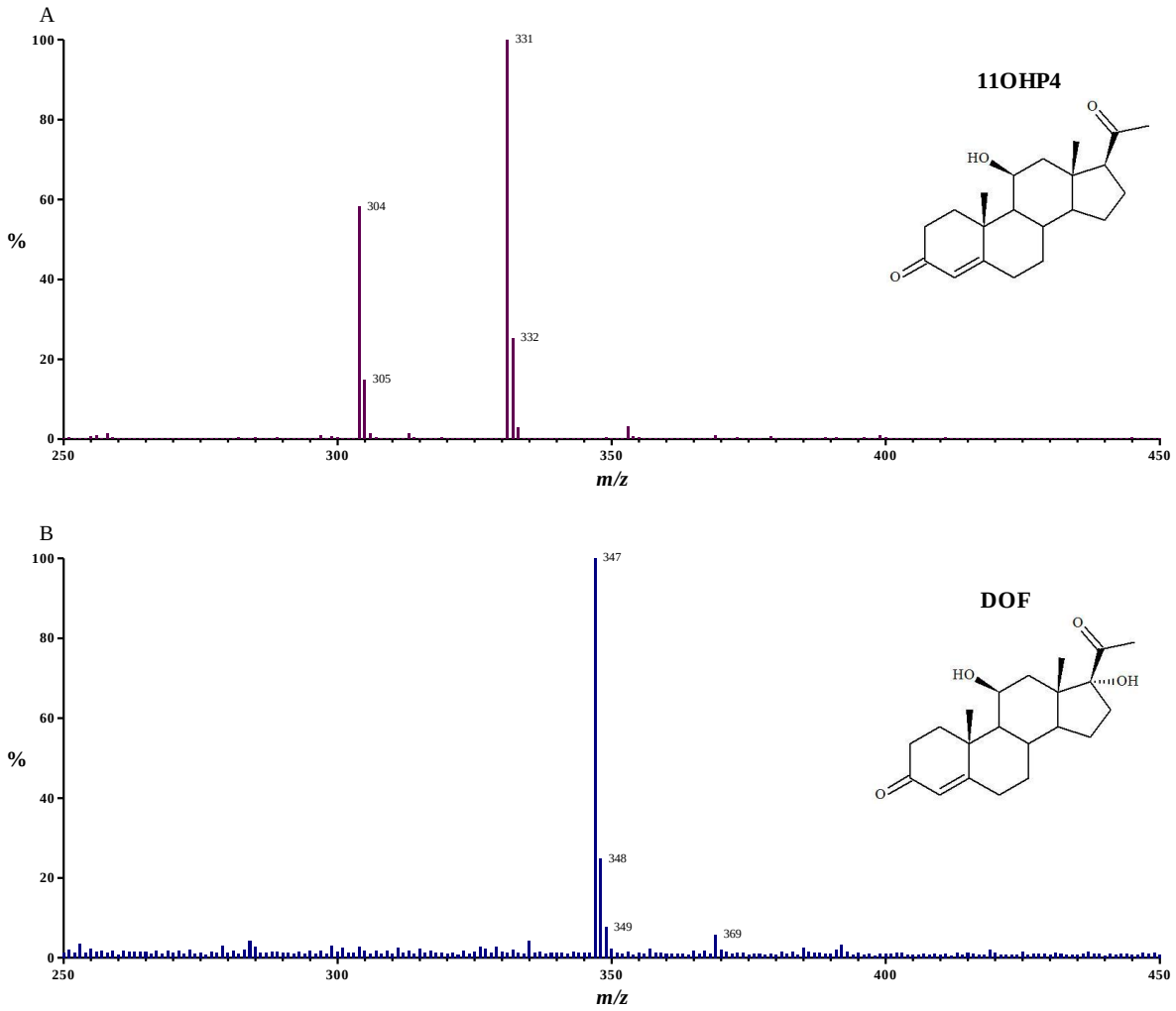


Figure 4.2 MS spectra obtained for (A) 11OHP4 ($[M+H]^+$ *m/z* 331.21) and (B) DOF ($[M+H]^+$ *m/z* 347.4). Data was acquired from *m/z* 150-600.

Table 4.3 Accurate mass determination of 11OHP4 and DOF. UHPLC retention times, accurate masses (observed and calculated), formulae and fragment ions are specified.

Compound	Retention time	Observed accurate mass $[M+H]^+$	Calculated mass	Formula	Fragments $[M+H]^+$
11OHP4	1.69	331.2269	331.2273	$C_{21}H_{31}O_3$	121; 295
DOF	1.22	347.2225	347.2222	$C_{21}H_{31}O_4$	97; 253

The fragmentation of 11OHP4 and DOF on the Q-tof MS/MS allowed the investigation and quantification of the 11β-hydroxylated products of P4 and 17OHP4 by the CYP11B isozymes on the UHPLC-MS/MS.

4.2.2.2 UHPLC-MS/MS analyses of steroid metabolites

UHPLC-MS/MS was employed for the analyses of the two steroid substrates, P4 and 17OHP4, together with their respective products 11OHP4 and DOF. The conversion of A4 to 11OHA4 was also assayed and used as a positive control. The internal standards D2-T and D9-P4 were included in the analyses. The chromatographic profiles of these steroids are depicted in Figure 4.3. MRM's for the detection of all steroids of interest could be achieved using the fragmentation spectrum obtained on the Q-tof-MS/MS, summarised in Table 4.4.

Table 4.4 UHPLC-MS/MS parameters for the separation of eight steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.

Steroid metabolite	RT (minutes)	Parent ion $[M+H]^+$	CV	Daughter ion[a] $[M+H]^+$	CE	Daughter ion[b] $[M+H]^+$	CE
P4	4.23	315.2	30	96.9	15	297.2	15
11OHP4	3.20	331.2	30	121	20	295.2	20
17OHP4	3.39	331.2	30	97	15	108.9	15
DOF	2.36	347.22	30	97	30	253	30
A4	3.27	287.2	30	96.9	15	108.8	15
11OHA4	2.40	303.2	30	121	30	267.2	15
D2-T	3.12	291	30	99.1	20	111.25	20
D9-P4	4.22	324.2	30	100	20	113	25

[a]Mass transition of the daughter ion used as quantifier

[b]Mass transition of the daughter ion used as qualifier

Using the parameters in Table 4.4, efficient separation and quantification of the six steroids and two internal standards were achieved in a single chromatographic step within a run time of 5 minutes. No prior clean-up or derivatisation was required. Some degree of cross-talk is observed between 11OHP4 and 17OHP4, due to the fact that these steroids share the same $[M+H]^+$ mass. Effective chromatoghrapic seperation is however achieved together with daugter ion selection according to MS/MS fragmentation.

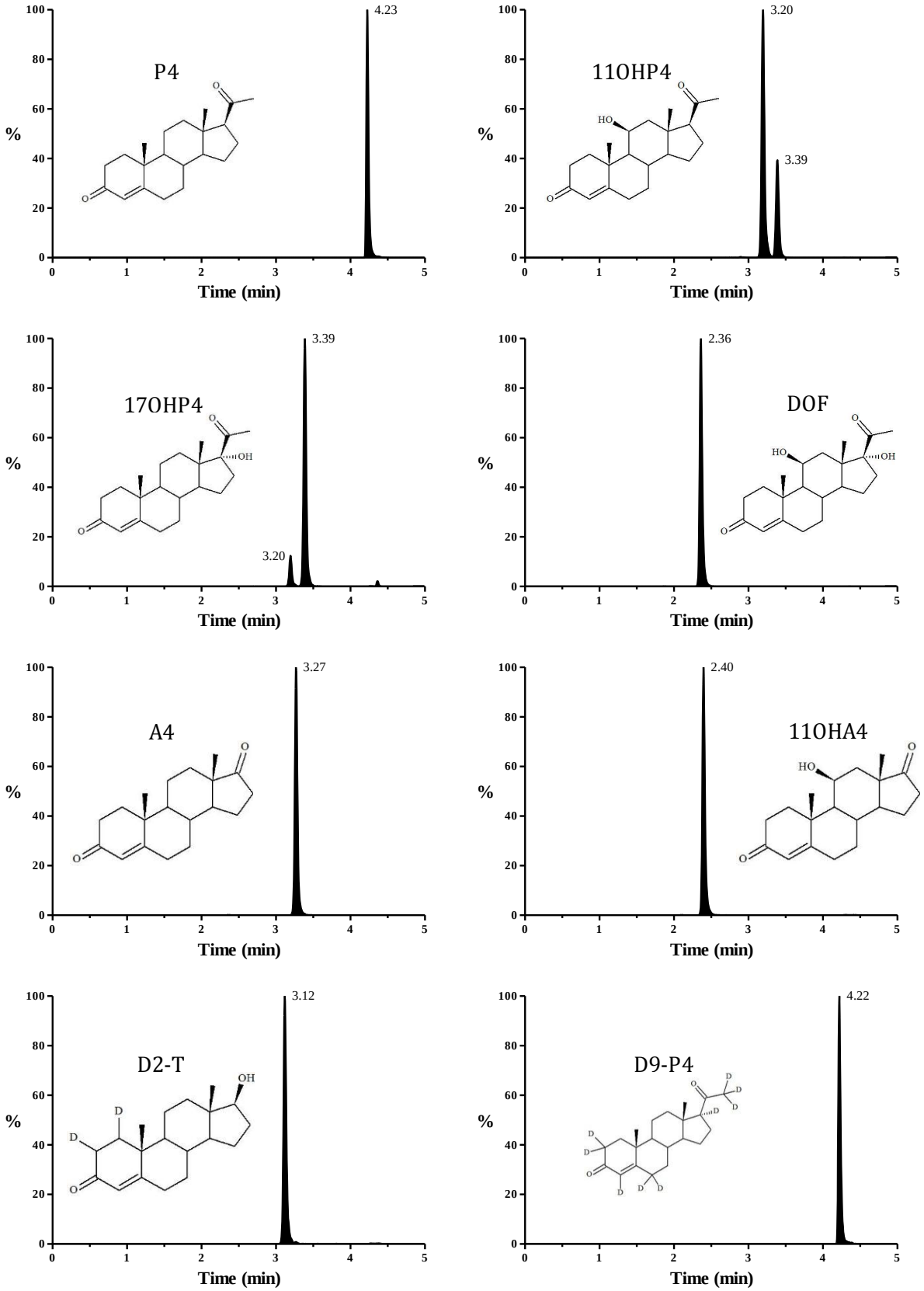


Figure 4.3 UHPLC-MS/MS chromatographic separation of eight steroid metabolites. Structures and retention times of steroids (shaded peaks) are indicated on each chromatogram. Chromatograms were generated during a 5 µL injection of an extracted 2 µg/mL steroid standard using MRM.

The development of an efficient UHPLC-MS/MS method that allowed the separation and quantification of the steroid metabolites of interest allowed further studies into these enzyme catalysed conversions. An example of the chromatographic separation multiplexed samples is shown in Figure 4.4.

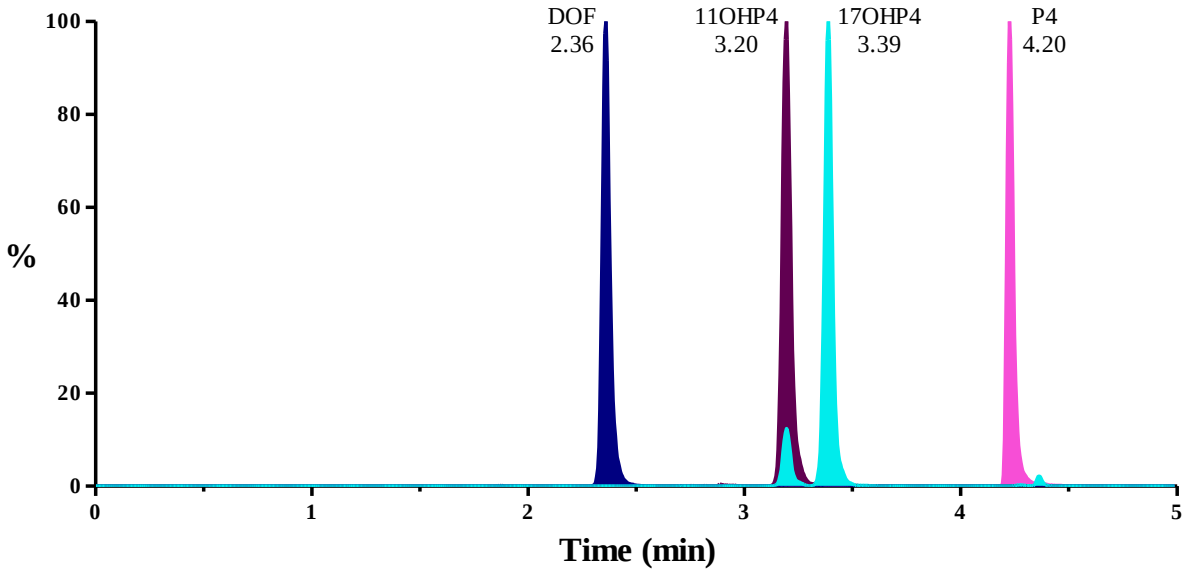


Figure 4.4 Overlaid UHPLC-MS/MS chromatogram of P4, 11OHP4, 17OHP4, and DOF. Retention times are indicated on the chromatogram with P4 eluting at 4.20 minutes, 11OHP4 at 3.20 minutes, 17OHP4 at 3.39 minutes and DOF at 2.36 minutes.

4.2.3 Biosynthesis of 11OHP4 and DOF in HEK293 cells expressing CYP11B

The metabolism of P4 was investigated over the course of 24 hours in HEK293 cells transiently expressing either CYP11B1 or CYP11B2 together with ADX. The chromatographic separation depicting the separation of the substrates and products in a single sample is shown above in Figure 4.4. CYP11B1 catalysed the reaction more readily than CYP11B2 (Figure 4.5), showing 50% conversion within 12 hours and reaching ±90% conversion after 24 hours. The catalytic conversion of P4 to 11OHP4 with CYP11B2 on the other hand merely reached ±60% conversion after 24 hours, with 50% conversion observed only after 16 hours. No significant difference in conversion between the two enzymes were observed for the first 12 hours, with the product formation only significantly different at 18 hours, displaying an even greater significant difference after 24 hours . Therefore, even though the conversion of P4 to 11OHP4 is initially similar, the conversion by CYP11B1 appears more favourable leading to significantly higher 11OHP4 levels after 24 hours, compared to the conversion by CYP11B2.

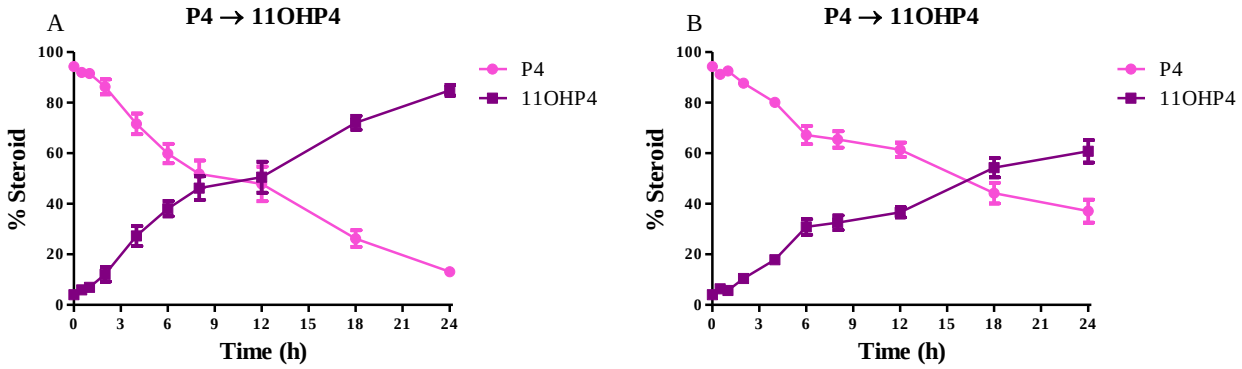


Figure 4.5 Metabolism of 1 µM P4 by CYP11B1 (A) and CYP11B2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM.

The metabolism of 17OHP4 was assayed in HEK293 cells transiently expressing CYP11B1 or CYP11B2 together with ADX over 24 hours. Conversion of 17OHP4 to DOF by CYP11B1 (Figure 4.6) reached ±70% conversion in 24 hours, with 50% conversion observed at 14 hours. CYP11B2 showed comparable conversion of 17OHP4 to DOF, with 50% conversion observed at 12 hours, reaching ±75% conversion after 24 hours. No apparent difference in catalytic capabilities was observed between the CYP11B isozymes towards 17OHP4, with comparable levels of DOF being produced even after 24 hours.

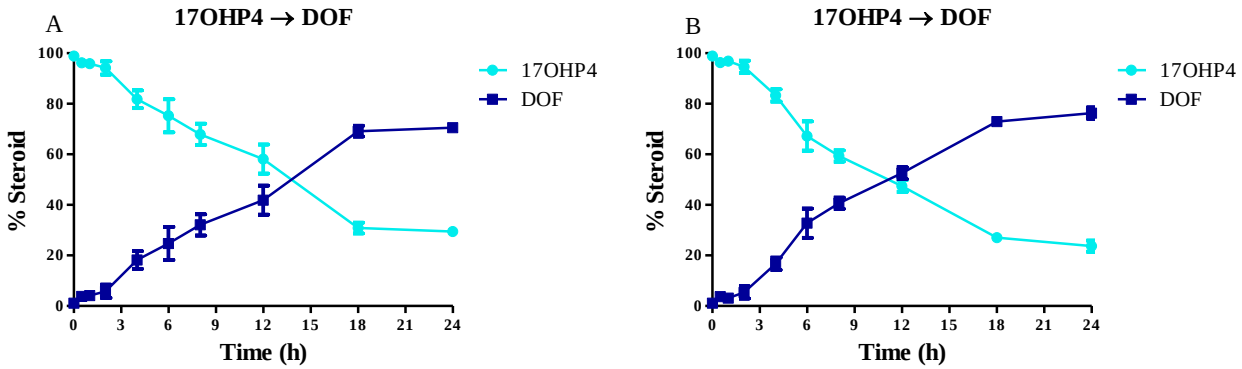


Figure 4.6 Metabolism of 1µM 17OHP4 by CYP11B1 (A) and CYP11B2 (B) in transiently transfected HEK293 cells over 24 hours. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM.

To establish effective transfection conditions, 1 µM A4, was assayed as a positive control to both CYP11B isozymes. Full conversion is observed with CYP11B1 at 24 hours while CYP11B2 only yielded 50% 11OHA4, consistent with previously published data (A. C. Swart et al., 2013).

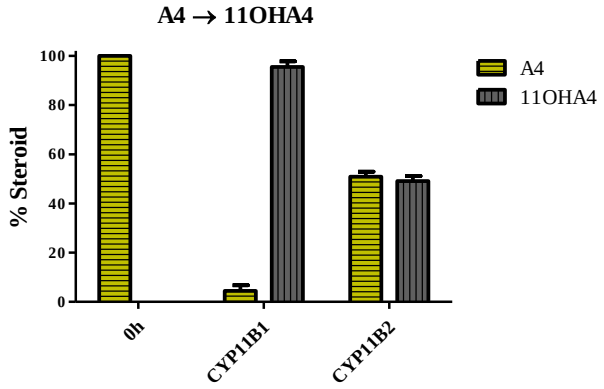


Figure 4.7 Metabolism of 1µM A4 by the CYP11B isozymes in transiently transfected HEK293 cells after 24 hours. Results represented as the mean ±SEM. 0h, sample aliquot collected at the start of the assay.

To ensure that the conversions demonstrated above was due to the expressed enzymes and not endogenous enzyme activity in the cells, HEK293 cells were transfected with the plasmid vector, pCIneo containing no inserted DNA. After 24 hours no significant conversion was observed with either substrate.

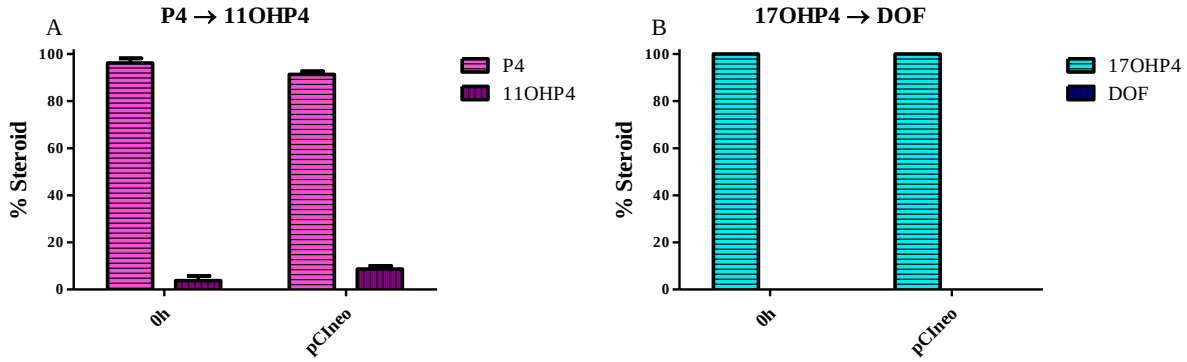


Figure 4.8 Metabolism of 1µM P4 (A) and 17OHP4 (B) in HEK293 cells after 24 hours. Results represented as the mean ±SEM. 0h, sample aliquot collected at the start of the assay; pCIneo, HEK293 cells transfected with vector without cDNA (negative control).

Comparing the catalytic activity of CYP11B1 and CYP11B2 towards P4 and 17OHP4 (Figure 4.9) statistical differences with P4 as substrate after 24 hours favouring the CYP11B1 isozyme was observed. No statistical significant differences were observed for the catalytic activity of the enzymes towards 17OHP4.

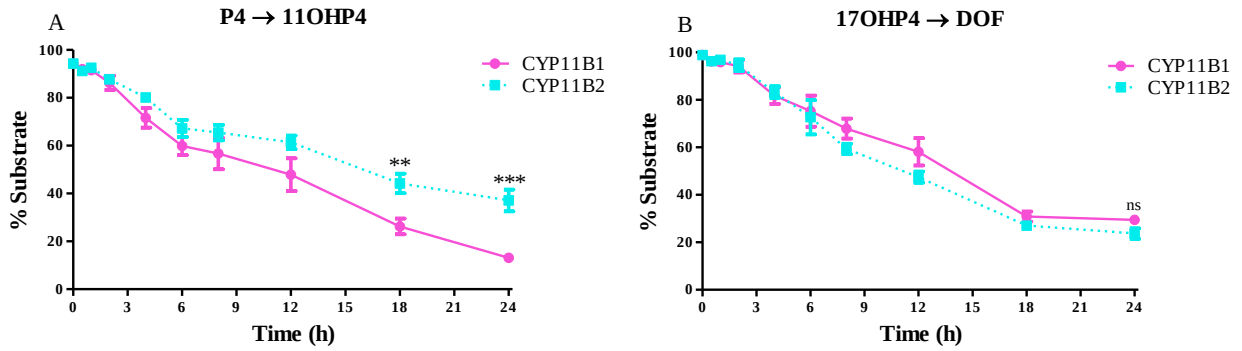


Figure 4.9 Comparison of the catalytic activity of the CYP11B isozymes towards 1µM P4 (A) and 17OHP4 (B). An unpaired t-test was performed and statistical significance was confirmed with a $P < 0.05$.

The catalytic activity of CYP11B1 towards P4 and 17OHP4 was also compared on a single graph, for a better visual representation, as well as the activity of CYP11B2 with P4 and 17OHP4 (Figure 4.10). CYP11B1 demonstrated a marginally higher catalytic activity towards P4 than 17OHP4 although significant at 24 hours (90% vs. 70%). The CYP11B2 enzyme showed a significantly greater conversion with 17OHP4 than P4 (75% vs. ±60%).

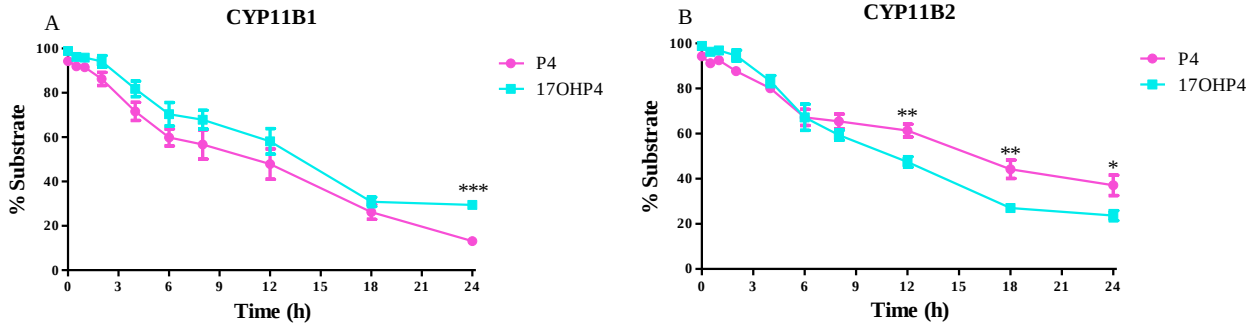


Figure 4.10 Comparison of the metabolism of 1µM P4 and 17OHP4 by CYP11B1 (A) and CYP11B2 (B). Statistical significance was determined using an unpaired t-test, with significance confirmed for $P < 0.05$.

Initially, no significant difference with CYP11B1 is observed, however after 24 hours a higher catalytic conversion of P4 is displayed, with significantly higher 11OHP4 production compared to DOF. Taking the overall conversion rates into account, a similar trend is observed and further investigation into initial rate kinetics are needed to draw definitive conclusions. In contrast the catalytic activity of CYP11B2 towards P4 and 17OHP4, favours the conversion of 17OHP4 towards DOF, producing significantly higher DOF after 12h. Once again similar trends in metabolism are observed for the first few hours and further investigation is still needed. Initial rates could not be calculated from the data presented in this study since the data represents two independent experiments.

Taken together, it is clear that both CYP11B1 and CYP11B2 has catalytic activity towards P4 and 17OHP4, catalysing the conversion towards 11OHP4 and DOF respectively.

4.3 Discussion

The adrenal specific expression of the CYP11B isozymes has been reported to be mainly involved in the biosynthesis of active mineralocorticoids and glucocorticoids, with the conversion of A4 to 11OHA4 believed to be an inactivation step (Goldzieher, de la Pena, & Aivaliotis, 1978). With the recent identification of the prominent role that the CYP11B enzymes have in the production of precursors to more potent steroid hormones and the major role they play in the progression of CRPC (A. C. Swart et al., 2013), interest into the potential substrates for these enzymes has grown. The discovery and elucidation of the 11OHA4 pathway (A. C. Swart et al., 2013; A. C. Swart & Storbeck, 2015) added a level of complexity to the production of potent androgens, that has since then been shown to contribute significantly to androgen dependant disease states (Rege et al., 2013). This led to the hypothesis that P4 and 17OHP4 may serve as additional substrates for the CYP11B isozymes, similar to A4 and T yielding 11OHA4 and 11OHT in the adrenal. This is particularly relevant in cases of P4 and 17OHP4 accumulation, as is seen in 21OHD, where these steroids are metabolised by downstream enzymes. The 11β-hydroxylation of P4 and 17OHP4, yielding 11OHP4 and DOF respectively may therefore add a new layer of complexity to adrenal steroidogenic diseases, especially when taking into account their downstream metabolism in steroidogenic tissue.

The catalytic activity of human CYP11B1 and CYP11B2 towards P4 and 17OHP4 was therefore investigated in this study. The CYP11B isozymes play a central role in the production of active mineralocorticoids and glucocorticoids in the adrenal, as well as the production of adrenal androgen precursors (Kawamoto et al., 1992; Storbeck et al., 2013). These enzymes usually catalyse the last steps within the three pathways and upstream products are not considered substrates for these enzymes. In 21OHD, the accumulation of P4, and more significantly 17OHP4, present possible substrates for the CYP11B isozymes since the defect in CYP21A2 prevents the production of adequate levels of the normal substrates, DOC and 11-deoxycortiol, in the mineralocorticoid and glucocorticoid pathways, respectively. The data demonstrated in this chapter clearly established that CYP11B1 and CYP11B2 hydroxylated P4 and 17OHP4 at C11, yielding 11OHP4 and DOF respectively. The accumulation of P4 and 17OHP4 in the 21OHD adrenal can therefore be shunted towards these steroid hormones. DOF, and to a lesser extent 11OHP4, has been identified in serum samples of 21OHD patients. The same group furthermore demonstrated the metabolism of P4 and 17OHP4 in TART cells, a cell model for 21OHD, yielded both 11OHP4 and DOF (Turcu et al., 2015), indicating that *in vivo* biosynthesis of these steroid hormones are possible. Future studies may therefore include the metabolism of P4 and 17OHP4 in the adrenal H295R cell model under basal and different stimulatory conditions (Forskolin, AngII and K^+). It may further be of

value to silence CYP21A2 mRNA, so that P4 and 17OHP4 metabolism can be investigated under conditions of limited CYP21A2 activity with accumulative P4 and 17OHP4 levels.

In this study the conversion of substrates at 1 µM P4 and 17OHP4, by CYP11B1 and CYP11B2 was compared in cells transiently expressing human enzymes and ADX cDNA plasmid constructs, establishing the biosynthesis of 11OHP4 and DOF by these enzymes. Analysis and accurate quantification was possible due to the implementation of a UHPLC-MS/MS method separating the steroids of interest in a single chromatographic step. Comparing the metabolism of P4 to 17OHP4, a higher catalytic activity towards P4 was observed with significantly higher 11OHP4 forming, compared to DOF, after 24 hours. P4 conversion to 11OHP4 by CYP11B1 reached ±90% after 24 hours, while only 70% DOF is produced. Similarly, CYP11B1 showed the higher catalytic activity towards P4 (90%) compared to 60% conversion obtained with CYP11B2. CYP11B1 expression is abundant in the zF, with cells also expressing CYP17A1 (Nishimoto et al., 2010; Suzuki et al., 2000). In the zF not only is the conversion of P4 to 17OHP4 thus observed, but the biosynthesis of 11OHP4 can produce a substrate for CYP17A1 that leads to the production of DOF. Therefore, even though CYP11B1 favours P4, the zone specific expression in the adrenal will not necessarily promote the formation of 11OHP4. Further studies can be focussed on the co-transfection of both CYP11B1 and CYP17A1 to establish the catalytic activity of these enzymes towards P4 when competing for the same substrate. As CYP11B1 is mainly involved in the production of glucocorticoids in the zF (Nishimoto et al., 2010), the zone specific expression in the adrenal thus favours 11-deoxycortisol as substrate for CYP11B1, producing the potent glucocorticoid, cortisol. The conversion of 11-deoxycortisol and DOC with CYP11B1 in transiently co-transfected COS cells, yields more than 90% cortisol and CORT respectively, within 6 hours. Additionally, the biosynthesis of ±80% 11OHA4 (6 hours) and ±65% 11OHT (16 hours) has been reported (A. C. Swart et al., 2013). Therefore, the catalytic activity of CYP11B1 is most efficient for 11-deoxycortisol and DOC. In the 21OHD adrenal, this is significant, since the 11-deoxycortisol and DOC formed during limited CYP21A2 activity, will be effectively utilised to produce cortisol and the mineralocorticoid precursor, CORT. In the absence of these substrates, P4, 17OHP4, A4 and T can compete for CYP11B1 – thus producing C11-oxy steroid metabolites.

The investigation of the metabolism of P4 and 17OHP4 by human CYP11B2 and ADX, showed that P4 and 17OHP4 are utilised by this enzyme, yielding 11OHP4 and DOF, respectively. Significantly higher conversion towards DOF is seen with CYP11B2 after 24 hours, when compared to 11OHP4 production. 75% 17OHP4 is converted to DOF, while P4 yielded 60% 11OHP4. Even though no significant preference is seen with 17OHP4 as substrate when the CYP11B isozymes are compared, the conversion by CYP11B2 is marginally higher, ±75%, compared to the ±70% conversion by CYP11B1. 17OHP4 therefore had the highest conversion by CYP11B2, which also exhibited the highest catalytic activity towards this steroid metabolite. CYP11B2 is exclusively expressed in the zG, where it is central in the production of mineralocorticoids, yielding ALDO (Nishimoto et al., 2010). CYP11B2 exhibits 11β-hydroxylase, 18-hydroxylase and 18-methyl oxidase activity, converting DOC to ALDO, via CORT and 18OHCORT

(Kawamoto et al., 1992). Our laboratory has recently showed that after 12 hours, CYP11B2 transiently expressed in COS cells, metabolised more than 90% DOC, yielding CORT (±30%), 18OHCORT (±50%) and ALDO (20%). In the same study, 11OHA4 and 11OHT production catalysed by CYP11B2 , after 12 and 16 hours was only 10% and 60%, respectively (A. C. Swart et al., 2013). Similar to CYP11B1, conversions with CYP11B2 is most efficient towards DOC, the natural substrate in the mineralocorticoid pathway. Once again, in the context of 21OHD, this will favour the immediate conversion of DOC to ALDO, during limited production of DOC. Since CYP11B2 is restricted to the zG, the abundant biosynthesis of P4 in this zone, place this steroid metabolite in close proximity to CYP11B2, making conversion towards 11OHP4 possible. The remaining substrates, 17OHP4, A4 and T would not be produced in the zG, due to the absence of CYP17A1 expression in this zone. However, circulating 17OHP4, of which high levels are present in 21OHD patients, may still reach the zG where it can be utilized by CYP11B2 to yield DOF. It is interesting to note that in comparison to CYP11B1, A4 and T were not catalysed as efficiently by CYP11B2 as the C_{21} steroids and as such, these androgens even at high circulatory levels would not be readily converted to their hydroxylated forms. This is evident from the control conversions with A4, shown in Figure 4.7, where full conversion with CYP11B1 was reached, but only ±50% conversion with the CYP11B2 enzyme was obtained. The resulting 11OHP4 produced in the zG can be secreted into circulation and may serve as a substrate for peripheral CYP17A1 leading to the biosynthesis of DOF. It is also possible that conversion by CYP17A1 at the zG and zF interface may produce limited amounts of DOF. This hypothesis is supported by studies that identified a segregated zone in the adrenal of 21OHD patients between the zF and zR that co-express 3βHSD2 and cytb5 (A. F. Turcu et al., 2016), placing the necessary enzymes for 17OHP4 production, 3βHSD2 together with CYP17A1 and cytb5 in close proximity to cells that express CYP11B isozymes. Similar segregated zones can exist in the adrenal of 21OHD patients that promote the production of 11- and 17-hydroxylated C_{21} steroids. The data presented thus far clearly shows the preference of CYP11B2 towards C_{21} steroids.

As mentioned before, the expression of CYP11B1 is regulated by ACTH, while the renin-angiotensin-aldosterone system regulates CYP11B2. Forskolin mimics the effect of ACTH, stimulating glucocorticoid production, resulting in a 6-fold increase in CYP11B1 expression in H295R cells after stimulation. Interestingly, the addition of forskolin also had a stimulatory effect on CYP11B2 (Oskarsson, Ullerås, Plant, Hinson, & Goldfarb, 2006). Foskolin has been reported to have a stimulatory effect in the production of steroids in the glucocorticoid and mineralocortiocoid pathways. The steroid metabolites CORT, 18OHCORT, ALDO and cortisol all increased upon forskolin stimulation (greater than 7-fold), possibly reflecting a stimulatory effect on the CYP11B isozymes by forskolin. ALDO had the highest fold increase upon stimulation (Schloms, Storbeck, Swart, Gelderblom, & Swart, 2012) and thus the role of CYP11B2 in steroid production stimulated by ACTH should not be overlooked. Regulation of ALDO production, and thus CYP11B2 expression, by the renin-angiotensin-aldosterone system has been reported to be independent of ACTH (Mihai, 2011). In 21OHD, the absence of cortisol's negative feedback on the HPA axis leads to increased ACTH levels, which stimulate the CYP11B isozymes, leading to increased

production of C11-oxy steroids. Similarly, activation of the renin-angiotensin-aldosterone system in an attempt to increase ALDO production, may therefore also lead to increased biosynthesis of 11OHP4 and DOF by the zone-specific production of P4 in the zG and increased circulating levels of 17OHP4 in 21OHD. Additionally, forskolin stimulation has also been shown to increase CYP17A1 activity (Rainey et al., 1993; Schloms et al., 2012). Taking into account that high ACTH levels are observed in 21OHD, this may increase activity of not only the conversion of P4 to 17OHP4, which can subsequently serve as a substrate for DOF production, but may also possibly convert 11OHP4 to DOF. This hypothesis is supported by studies undertaken by Turcu, *et al*. identifying 11OHP4 in TART cells during basal conditions, but did not detect 11OHP4 after ACTH stimulation. ACTH stimulation however also increased DOF biosynthesis (Turcu et al., 2015), suggesting the stimulatory role of ACTH on CYP17A1, directing the steroid flux towards DOF.

Unpublished data by our laboratory has further confirmed that the C11-oxy C_{21} steroids are substrates for the 11βHSD isozymes. Both 11OHP4 and DOF are converted to their respective C11-keto products with 11βHSD2, yielding 11KP4 and DOE respectively. A higher catalytic activity was seen for DOF, yielding almost 80% DOE, while 11OHP4 did not reach 50% conversion after 24 hours. Higher conversions of cortisol and 11OHA4 were observed with 11βHSD2, yielding cortisone and 11KA4 respectively. With the 11βHSD1 isozyme 11KP4 and DOE were converted to 11OHP4 and DOF respectively, with a higher conversion of 11KP4. Both steroids showed more than 50% conversion after 24 hours, but the conversion of 11KA4 and cortisone, yielding 11OHA4 and cortisol, still showed precedence. Taken together this suggest that the 21OHD adrenal has the ability to produce the C11-oxy C_{21} steroids, 11OHP4, 11KP4, DOF and DOE, with the 11βHSD isozymes favouring 11OHP4 and DOE biosynthesis.

In conclusion, the CYP11B isozymes exhibit catalytic activity towards P4 and 17OHP4, which is present at high levels in the 21OHD adrenal. ACTH stimulation will lead to increase activity of not only the CYP11B isozymes but also CYP17A1. This increased activity together with the 11βHSD activity in the adrenal will favour the production of C11 and C17 hydroxylated C_{21} steroids, supported by high DOF levels observed in adrenal vein samples (A. F. Turcu et al., 2015). In the next chapter, the downstream metabolism of DOF and DOE will be investigated.

CHAPTER 5

THE METABOLISM OF DOF AND DOE

The human adrenal is a prominent site for the biosynthesis of several C_{19} steroids, including A4, DHEA, DHEA-S, and 11OHA4 (Miller & Auchus, 2011; Rege et al., 2013). Even though these steroids exhibit limited androgenic activity, they contribute to the pool of circulating adrenal androgen precursors that may be converted to more potent androgens in peripheral target tissue. In 21OHD, an increase in the flux towards the adrenal androgen precursors is observed and recent studies have found that the C11-oxy C_{19} steroids, 11OHA4, 11KA4, 11OHT and 11KT levels were significantly higher in adrenal vein samples of 21OHD patients (A. F. Turcu et al., 2016). In addition, 21OHD patients present with high levels of the 21-hydroxylase substrates, P4, 17OHP4 and DOF (A. F. Turcu et al., 2015). The adrenal enzymes CYP11B1 and CYP11B2 have now been shown to use P4 and 17OHP4 as substrates to produce 11OHP4 and DOF as discussed in the previous chapter.

Our laboratory recently unravelled the 11OHA4 pathway and highlighted the importance of the C11-oxy C_{19} steroids in the prostate. Noteworthy is the production of 11KDHT, a potent androgen, with AR agonist activity comparable to DHT (Bloem et al., 2015; Storbeck et al., 2013). In addition, the backdoor pathway described the production of the potent androgen DHT from 17OHP4. The biosynthesis of 17OHP4 in the adrenal may therefore not only contribute to the production of potent glucocorticoids, but also to the production of the potent androgen DHT. In this chapter we propose that in the case of 21OHD, the biosynthesis of DOF and DOE in the adrenal may add to the production of C11-oxy androgen metabolites, specifically 11OHDHT and 11KDHT, which may contribute significantly to the classic symptoms of androgen excess associated with 21OHD. DOF and DOE may be metabolised by the same enzymes as in the case of 17OHP4, ultimately leading to the production 11OHDHT and 11KDHT, respectively (Figure 5.1).

The backdoor pathway presents an alternative route in the production of androgens in cases of accumulating 17OHP4, as is observed in 21OHD. Since limited CYP17A1 lyase activity is observed towards 17OHP4 (Imai et al., 1993), the backdoor pathway presents substrates other than the conventional 17OHP5 and 17OHP4 substrates for the lyase activity of CYP17A1. Preliminary studies in our laboratory have found that DOF and DOE, in a similar manner to 17OHP4, do not yield significant conversion towards 11OHA4 and 11KA4 in assays with human CYP17A1 (unpublished data). The elevated production of DOF, and possibly DOE, in 21OHD patients must therefore be metabolised in an alternative manner. In the backdoor pathway the conversion of these steroid metabolites by SRD5A and AKRIC2, yielding 5α-pregnan-3α, 11β, 17α-triol-20-one (11OHPdiol) and 5α-pregnane-3α, 17α-diol-11, 20-dione (11KPdiol) respectively, may present possible substrates for the lyase activity of CYP17A1. It is therefore possible that in 21OHD, the

production of C11-oxy C_{21} steroid precursors, may become as important as the production of C_{19} steroid precursors in these patients in the downstream production of potent androgens in peripheral tissue. DOF has furthermore been reported to be an excellent marker for 21OHD, able to distinguish between 21OHD carriers and NC 21OHD patients (Fiet et al., 2000; Milewicz et al., 1984), with the identification of novel steroid metabolites thus contributing to disease classification.

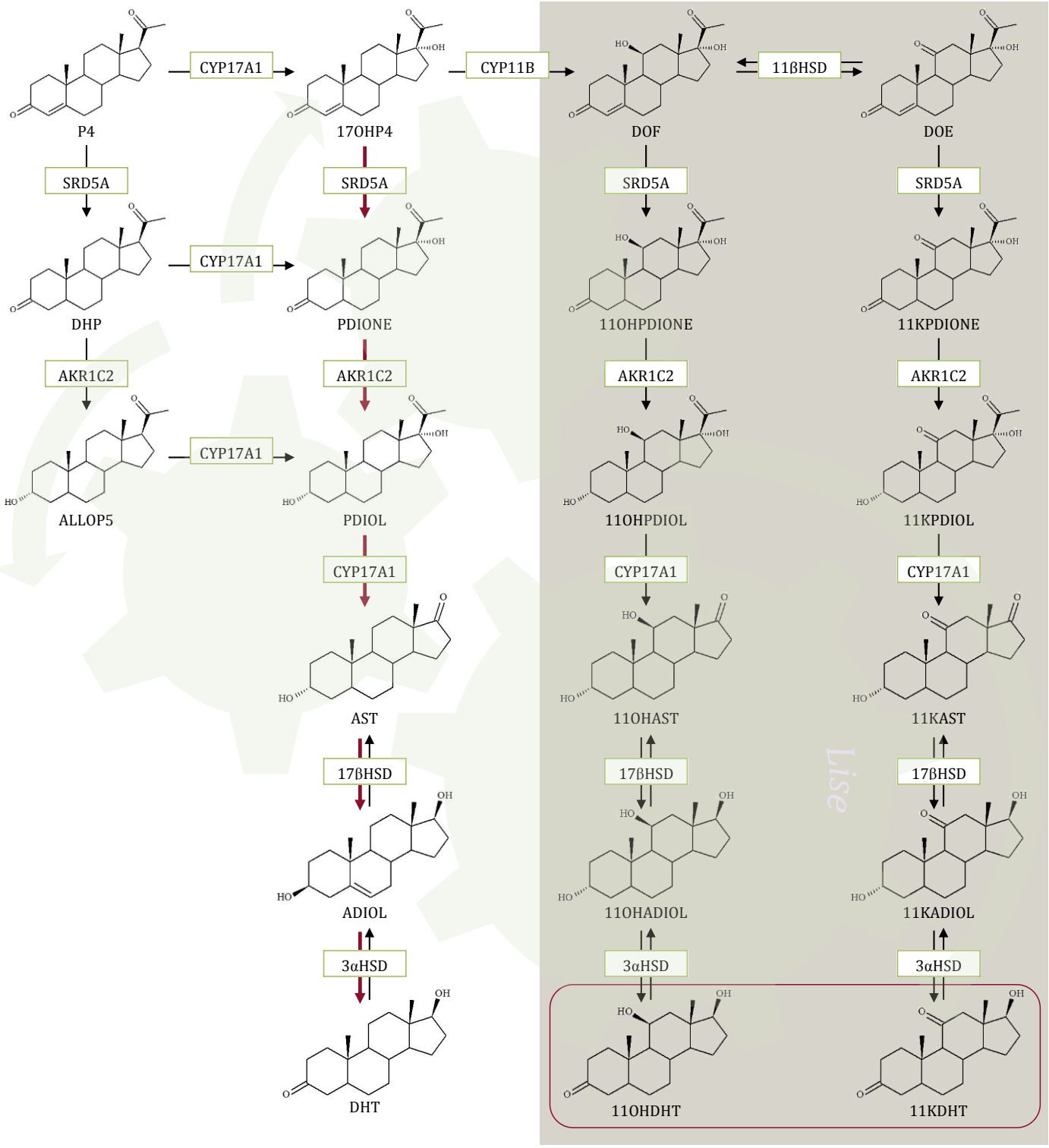


Figure 5.1 Production of DHT in the backdoor pathway (red arrows). The proposed metabolism of DOF and DOE and the biosynthesis of 11OHDHT and 11KDHT (boxed steroids) are indicated in the shaded area.

The aim of this investigation was to determine the downstream metabolism of DOF and DOE catalysed by the enzymes characteristic of the backdoor pathway - conversion of DOF and DOE by the SRD5A isozymes and AKR1C2. Furthermore we demonstrate 17OHP4 and DOF metabolism in prostate cell models.

5.1 Methodology

5.1.1. Materials

T and DHT, MTBE, β-glucuronidase (Type-VII-A from *E.coli*; 5292000 U/g), D-(+)-glucose, RPMI 1640, DMEM and Ham's F12K media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Steraloids (Winston, USA) supplied P4, DHP, Pdione, Pdiol, 17OHP4, DOF, DOE and DHT-G. Deuterated steroids D2-T, D7-A4 (4-androsten-3,17-dione 2,2,4,6,6,16,16-D7, 98%), D7-11OHA4 (4-androsten-11β-ol-3,17-dione 2,2,4,6,6,16,16-D7, 98%) and D9-P4 were obtained from Cambridge Isotopes (Andover, MA, USA). Wizard® Plus Midipreps DNA purification system was supplied by Promega Biotech (Madison, WI, USA). Corning® Cellbind® Surface 24 well and 100 mm plates were purchased from Corning® Life Sciences (Corning, NY, USA). XtremeGene HP® DNA transfection reagent was supplied by Roche Diagnostics (Mannheim, Germany). FBS, penicillin-streptomycin and trypsin-EDTA were bought from Gibco BRL (Gaithersburg, MD, USA). HEPES and sodium pyruvate were obtained from Biochrom (Berlin, Germany). pH indicator strips (non-bleeding) were supplied by Merck (Darmstadt, Germany). Trypan blue stain (0.4%) together with cell count plates were acquired from Invitrogen (Eugene, USA). All other reagents were supplied by trustworthy scientific supply houses.

5.1.1.1 Cell lines and vectors

HEK293 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA) and LNCaP, PNT2 and PC3 cells were obtained from Sigma's European Collection of Cell Cultures (St. Louis, MO, USA). HEK293 cells were cultured in DMEM, LNCaP and PNT2 cells in RPMI 1640 media, with PC3 cells cultured in Ham's F12K media. Media was supplemented with 1.5 g $NaHCO_3$, 10% FBS and 1% penicillin-streptomycin, with LNCaP media additionally supplemented with D-(+)-glucose (2.5 g/L) and 5 mL of HEPES (1 M) and sodium puruvate (1 M) each. The culture environment was kept stable at 37 °C, 5% CO_2 and 90% relative humidity. Cells were kept in cryogenic storage until needed and then allowed three passages before use in experiments. All cells tested negative for mycoplasma on the third passage cycle. Confluent cells were plated at a concentration of $1x10^5$ cells/mL during normal passage. Prof DW Russell donated the pCMV7/SRD5A1 and pCMV7/SRD5A2 plasmid constructs while the pcDNA3/AKR1C2 vector was a kind gift from Prof TM Penning.

5.1.2 Purification of plasmid DNA

Freezer stocks of *E.coli* cells (strain JM109) containing the plasmid vectors pCIneo, pCMV7/SRD5A1, pCMV7/SRD5A2 or pcDNA3/AKR1C2 were streaked out on ampicillin containing (100 µg/mL) LB-agar plates. Single colonies were selected and inoculated in 5 mL of LB-media containing 0.1% ampicillin (100 mg/mL) and incubated for 8 hours at 37 °C. The starter culture was diluted by pipetting 100 µL culture to 100 mL of fresh LB-media containing 0.1% ampicillin (100 mg/mL). The culture was placed on a shaking incubator (Innova; New Brunswick, Canada) at 225 rpm and 37 °C until the cell culture reached an OD_{600} of 1 (±16 hours). Plasmid cDNA (human) was purified using Wizard® *Plus* Midipreps DNA Purification System as set out by the manufacturer and stored at -20 °C until needed. DNA plasmid concentrations and 260/280 ratios were determined using UV spectrophotometry (Cary60 UV-Vis by Agilent technologies; Santa Clara, CA, USA).

5.1.3 Enzyme assays in transiently transfected HEK293 cells

HEK293 cells were cultured in supplemented DMEM (10% FBS, 1% penicillin-streptomycin) in a stable environment at 37 °C, 5% CO_2 and 90% relative humidity as described in *section 5.1.1.1*. Cells used for experiments were seeded at a cell density of $2x10^5$ cells/mL in 100 mm Corning® CellBIND® surface plates and 24 well Corning® CellBIND® surface plates. Within 24 hours after cells were plated, the cells were transiently transfected with 0.5 µg plasmid cDNA per well and 11 µg plasmid cDNA per 100 mm plate using XtremeGene DNA transfection reagent according to the manufacturer's instructions. For studies assaying 5α-reductase activity, pCMV7/SRD5A1 and pCMV7/SRD5A2 was used with the pCIneo vector without insert cDNA as negative control. Transiently transfected cells were incubated for a further 48 hours, where after the media was aspirated and replaced with media (12 mL per 100 mm dish and 600 µL per well) containing 5 µM of the appropriate steroid (P4, 17OHP4, DOE, DOF or T). Time points (500 µL aliquots) were collected in triplicate over the course of 12 hours and stored at 4 °C until steroid extraction. For the analyses of steroids in conversion assays by AKR1C2, the substrates (5α-pregnan-17α-ol-3, 11, 20-trione (11KPdione) and 5α-pregnan-11β, 17α-diol-3, 20-dione (11OHPdione)) were not commercially available and were thus produced in SRD5A2 transfected cells after which the media was to be transferred to AKR1C2 transfected cells. Therefore, 48 hours after transfection with SRD5A2 the media was replaced with 600 µL media containing the appropriate steroid (DOF or DOE). In parallel, fresh confluent cells were replated and transiently transfected after 24 hours with 0.5 µg AKR1C2 plasmid cDNA per well. The media was transferred from the SRD5A transfected cells after 12 hours, to cells transiently expressing pcDNA3/AKR1C2 or pCIneo (negative control). Aliquots, 500 µL were also taken at this time for UHPLC-MS/MS analyses to verify full conversion of substrates by SRD5A2. Following a further 24 hours 500 µL triplicate aliquots were collected and stored at 4 °C until steroid extraction.

5.1.4 DOF and DOE metabolism in LNCaP, PNT2 and PC3 cells

LNCaP and PNT2 cells were cultured in supplemented RPMI 1640 media (10% FBS, 1% penicillin-streptomycin and 1.5 g/L $NaHCO_3$) with LNCaP media additionally supplemented with 2.5 g/L D-(+)-glucose, 1% HEPES and 1% sodium pyruvate. PC3 cells were cultured using Ham's F12K media supplemented with 10% FBS, 1% penicillin-streptomycin and 1.5 g/L $NaHCO_3$. All cells were kept in a stable environment at 37 °C, 5% CO_2 and 90% humidity. After 3 passages cells were seeded at $2x10^5$ cells/mL and plated in 24 well Corning® CellBIND® surface plates. After 48 hours the media was aspirated and replaced with 500 µL fresh media containing the appropriate steroid (1 µM 17OHP4 or DOF). Following a further 24 hours, 500 µL time points was taken in triplicate and stored at 4 °C until steroid extraction. LNCaP samples were treated with 400 units of β-glucuronidase (*section 5.1.4.1*) prior to steroid extraction.

5.1.4.1 Deconjugation

Steroid extraction procedures carried out prior to ultra-performance convergence chromatography-tandem mass spectrometry (UPC^2-MS/MS) analyses isolates only free unconjugated steroids and since LNCaP cells express high levels of UGT2B15 and UGT2B17 enzymes (Chouinard, Barbier, & Bélanger, 2007), steroid substrates with a hydroxyl-group on C3 or C17 may be conjugated. Before extraction a deconjugation reaction was thus carried out on samples collected in the assay conducted in LNCaP cells, according to the manufacturer's instructions. Samples were incubated for 1 hour at 37°C after which the pH was set to 6.5 using non-bleeding pH indicator strips by adding 1% acetic acid (± 23 µL) to each sample. Thereafter 400 units of β-glucuronidase (*E.coli* Type VII-A) was added, samples thoroughly mixed and incubated for 24 hours at 37°C. Following incubation steroids was immediately extracted using liquid-liquid extractions.

5.1.5 Steroid extraction

Samples were retrieved from 4 °C and allowed to reach room temperature. 100 µL internal standard (15 ng of D2-T, D7-A4, D7-11OHA4 and/or D9-P4) and 1.5 mL MTBE was added to each sample. Samples were vortexed for 10 minutes and placed at -80 °C for 1 hour. The resulting liquid organic phase was transferred to a clean labelled test tube and dried under a stream of nitrogen at 45 °C. Samples were resuspended in 95 µL methanol (100%) and transferred to a MS vile insert. Equal amounts of MilliQ water was added to samples to ensure 50% MeOH, compatible with the LC-MS/MS systems.

5.1.6 LC-MS/MS analyses of steroid metabolites

5.1.6.1 Accurate mass determination of novel C11-oxy C_{21} steroids

Novel steroid substrates were assayed during the conversions of DOF and DOE by SRD5A. To determine successful conversion the accurate mass of steroid metabolites was established using a Waters Synapt G2 Q-tof-MS (Waters Corporation, Milford, MA, USA) coupled to an ACQUITY UHPLC (Waters

Corporation, Milford, MA, USA). A BEH C_{18} column (2.1 x 100 mm) was used in the UHPLC and the MS conducted in ESI^+ mode using MRM. Instrumental parameters as follow: source temperature, 120 °C; sampling cone voltage, 15 V; extraction cone voltage, 4 V; desolvation temperature, 275 °C with a desolvation gas flow, 650 L/hour; cone gas flow, 50 L/hour; capillary voltage, 2.5 kV; collision energy, 4 eV; column temperature, 40 °C, with the acquisition mass for MS ranging from 120-600 *m/z* and for MS/MS ranging from 40-800 *m/z*. Leucine enkephalin was used as reference compound with a lock mass of 556.2771 *m/z*. A total run time of 7 minutes was allowed with a 5 µL injection volume for MS analyses and 10 µL injection volume for MS/MS analyses. Formic acid (1%) in deionised water was used as solvent A1 with acetonitrile as solvent B1 with a flow rate of 0.35 mL/minute and the solvent make-up as follow: the run began with 98% solvent A1, kept stable over the first 6 seconds, after which a linear gradient up until 5 minutes was followed to reach a 10:90 ratio of A1:B1. This ratio was kept stable for 30 seconds, with the next 30 seconds following a linear gradient to return to the initial parameters that were kept stable for the remainder of the run. 1000 µL water was used as a weak solvent wash with 500 µL B1 as strong solvent wash. All analyses were carried out on the MassLynx v4.1 software package.

5.1.6.2 UHPLC-MS/MS separation and quantification of steroid metabolites

Steroid analytes were analysed on the UHPLC-MS/MS using an ACQUITY UHPLC (Waters Corporation, Milford, MA, USA) coupled to a QTMS (Waters Corporation, Milford, MA, USA). Steroid standards, 2 mg/mL, P4, DHP, 17OHP4, Pdione, DOF, DOE, T and DHT powder were dissolved in absolute ethanol. A standard range (0.02-2.5 ng/µL) was prepared in DMEM culture media and 500 µL aliquots were extracted according to the protocol in *section 5.1.5*. Steroid metabolites were separated on a Kinetex PFP column (2.1 mm x 100 mm x 2.6 µm) (Phenomenex Corporation, Torrance, CA, USA) before introduction on the MS. Analyses on the QTMS were ran in ESI^+ mode using MRM. Instrumental parameters as follow: capillary voltage, 3.5 kV; source temperature, 140 °C; desolvation temperature, 400 °C and desolvation gas flow, 800 L/hour[2]; cone gas flow, 50 L/hour; collision gas flow, 0.2 mL/minute; collision energy, 4 eV and column temperature, 50 °C with sample temperature, 15 °C. A total run time of 5 minutes was allowed using a mobile phase consisting of a mixture of methanol:acetonitrile:isopropanol (49:49:2) as solvent A1 and 1% formic acid in deionised water as solvent B2. Mixture make-up and gradient is summarised in table 5.1. Injection volume was set at 5 µL at a flow rate of 0.4 mL/minute. A mixture of water (90%), acetonitrile (10%) and formic acid (0.01%) was use as a weak solvent wash (1000 µL) and an acetonitrile and formic acid (0.01%) mixture for the strong solvent wash (500 µL). MassLynx v4.1 Software package was used for data collection and analyses.

[2] For the analyses of AKR1C2 conversion desolvation gas flow was set to 750 L/hour.

Table 5.1 UHPLC solvent parameters for the chromatographic separation.

	Time (minutes)	Flow rate (mL/minute)	Solvent A (%)	Solvent B (%)	Curve gradient
1	Initial	0.400	85	15	0
2	0.50	0.400	55	45	6
3	3.50	0.400	35	65	6
4	3.60	0.400	0	100	6
5	4.00	0.400	0	100	6
6	4.01	0.400	85	15	6
7	5.00	0.400	85	15	6

5.1.6.3 Separation and quantification of steroid metabolites using UPC²-MS/MS

A novel method was set-up on the AQUITY UPC^2-MS/MS (Waters Corporation, Milford, USA) to investigate the presence of novel steroid metabolites in cell models. Standards (2 mg/mL), 17OHP4, Pdione, Pdiol, AST, DOF, 11OHAST, DOE, 11KAST and DHT was prepared by dissolving steroid powder in absolute ethanol and used to prepare a standard range of 1-1000 ng/mL in culture media. Steroids were extracted as described in *section 5.1.5*. Samples (2 µL) were injected unto an ACUITY UPC^2 BEH (3.0 mm x 100 mm, 1.7 µm) column (Waters Corporation, Milford, USA), to allow chromatographic separation before introduction to the Xevo TQ-S triple quadrupole mass spectrometer (Water Cooperation, Milford, USA). Steroids were analysed in ESI$^+$ mode using MRM. Instrumental parameters as follow: capillary voltage, 3.70 kV; cone voltage, 20 V; cone gas flow, 150 L/hour; source temperature, 150 °C; column temperature, 60 °C; desolvation temperature, 500 °C; desolvation gas flow, 900 L/hour, collision gas flow, 0.15 mL/minute; MS collision energy, 10 eV and MS/MS collision energy, 5 eV. The system pressure was regulated with the automated back pressure set at 2000 psi. A make-up pump mixed methanol with 1% formic acid (Solvent B), flowing into the system at a constant rate of 0.2 mL/minute. The mobile phase consist out of liquid CO_2 (Solvent A) modified with Solvent B as set out in Table 5.2.

Table 5.2 UPC^2 solvent parameters for the chromatographic separation.

	Time (minutes)	Flow rate (mL/minute)	Solvent A (%)	Solvent B (%)	Curve gradient
1	Initial	2.000	98	2	Initial
2	0.50	2.000	98	2	6
3	3.50	2.000	85	15	6
4	4.50	2.000	98	2	6

5.1.7 Statistical analyses

GraphPad Prism 5 was used for all statistical analyses. Experiments were carried out in triplicate and the results displayed as the mean value ±SEM. Unpaired t-test or one-way ANOVA with a Dunette's post-test or Turkey post-test was used to determine statistical significance. P-values < 0.05 were taken as a significant difference.

5.2 Results

Since it has been shown that the adrenal steroid metabolite, 17OHP4, can be metabolised to DHT, in the backdoor pathway this study set about investigating the metabolism of the C11-oxy metabolites DOF and DOE, by the first two enzymes in the backdoor pathway, SRD5A and AKR1C2.

5.2.1 Plasmid cDNA analyses

Plasmid DNA was prepared and purified to investigate the catalytic conversions of the SRD5A isozymes and AKR1C2. Table 5.3 summarises the average DNA yield obtained from a 100 mL *E. coli* culture as well as the 260/280 ratio as measured on the Cary60 UV-Vis (Agilent Technologies, Santa Clara, CA, USA). A ratio ranging from 1.8-2 is optimal, since values lower than this is indicative of protein contamination, while values above 2 indicate possible RNA contamination. Purified plasmid DNA was used in the heterologous expression of the relevant enzymes in transfected cell model systems.

Table 5.3 Plasma yields obtained from DNA purification

cDNA	Yield (µg)	Ratio
pCMV7/hSRD5A1	254.8	1.85
pCMV7/hSRD5A2	249.3	1.84
pcDNA3/hAKR1C2	190.0	1.82
pCIneo	454.6	1.84

5.2.2 Metabolism of DOF and DOE by SRD5A

SRD5A catalysed conversions, are the first metabolic or activating step in the backdoor pathway. As mentioned previously, the metabolism of DOF and DOE by the enzymes in the backdoor pathway has not been demonstrated.

5.2.2.1 Accurate mass determination of 11OHPdione and 11KPdione

No steroid standards were available for the novel C11-oxy C_{21} steroids, 11OHPdione and 11KPdione, and therefore their identity was determined by accurate mass determination (Figure 5.2) using a Waters

Synapt G2 Q-tof. Once the novel C11-oxy C_{21} steroids were identified, further fragmentation with Q-tof-MS/MS was carried out. Parent ions were selected at their specific $[M+H]^+$ mass during the first round of MS and further fragmented in a coupled collision cell before the second round of MS was performed. This resulted in a wide range of product ions for each specific parent ion. Daughter ions with high signal intensity and specificity were used for the MRM set-up on the UHPLC-MS/MS. Accurate masses for the novel C11-oxy C_{21} steroids are summarised in Table 5.4.

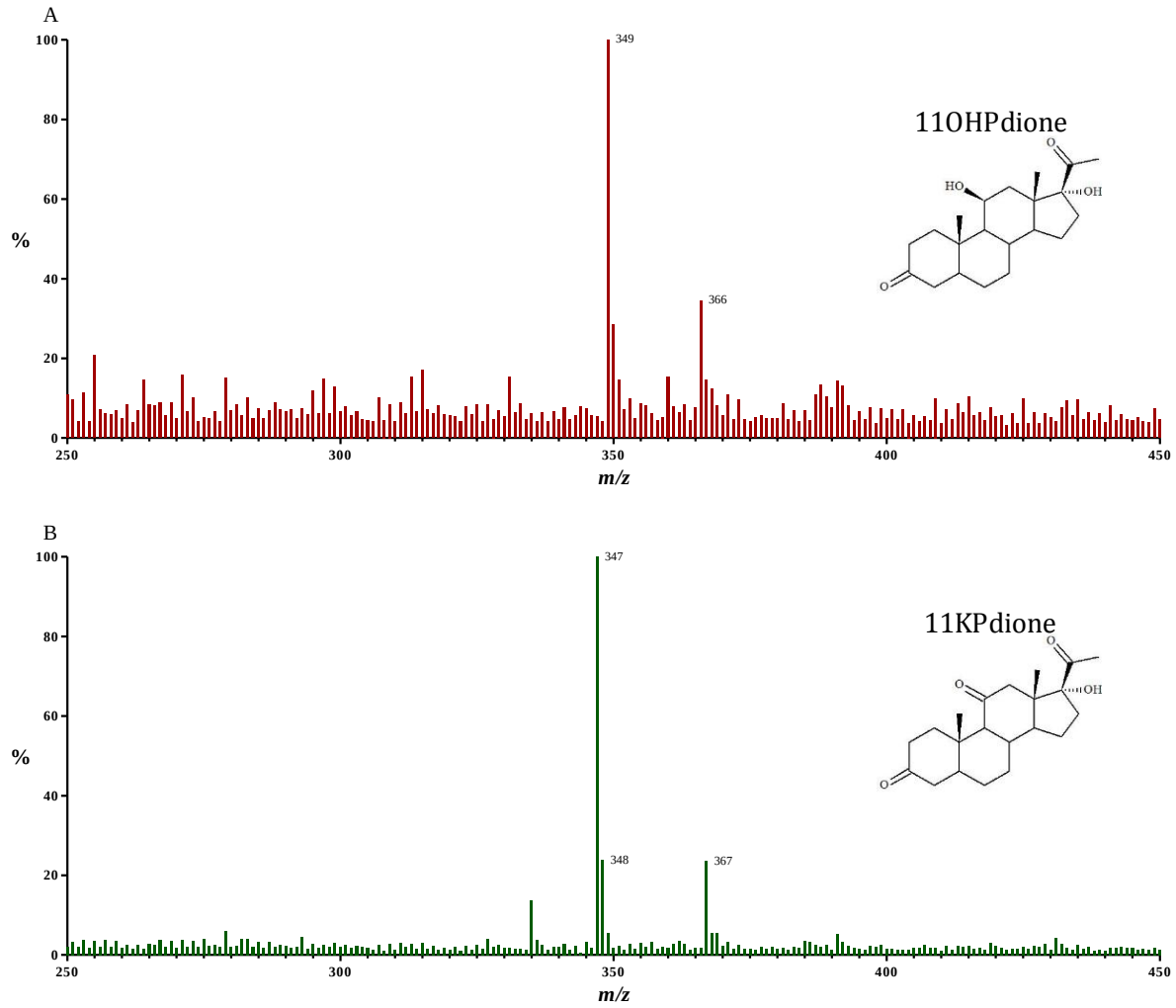


Figure 5.2 MS spectra of (A) 11OHPdione ($[M+H]^+$ *m/z* 349.4) and (B) 11KPdione ($[M+H]^+$ *m/z* 347.4). Acquired mass range was scanned across *m/z* 150-600.

Table 5.4 Accurate mass determination of 11OHPdione and 11KPdione. Accurate masses (observed and calculated), formulae and fragment ions are depicted.

Compound	Observed accurate mass $[M+H]^+$	Calculated mass	Formula	Fragments $[M+H]^+$
11OHPdione	349.2133	349.2379	$C_{21}H_{32}O_4$	253
11KPdione	347.2218	347.2222	$C_{21}H_{30}O_4$	147, 269, 329.2

5.2.2.2 UHPLC-MS/MS analyses of steroid metabolites

The Q-tof-MS/MS accurate mass determination and fragmentation of the C11-oxy C_{21} steroid metabolites permitted further UHPLC-MS/MS analyses. The catalytic conversion of the known steroid substrates, P4 and 17OHP4, together with DOF and DOE by the SRD5A isozymes was analysed using UHPLC-MS/MS. The conversion of T to DHT was used as positive control (Figure 5.11) with D2-T, D7-A4, D7-11OHA4 and D9-P4 as internal standards. The accurate mass determination and further fragmentation on the Q-tof-MS/MS was used to set-up MRM's for the novel C11-oxy C_{21} steroids. A UHPLC-MS/MS method for the investigation of SRD5A conversions was established with the parameters used for the separation summarised in Table 5.5.

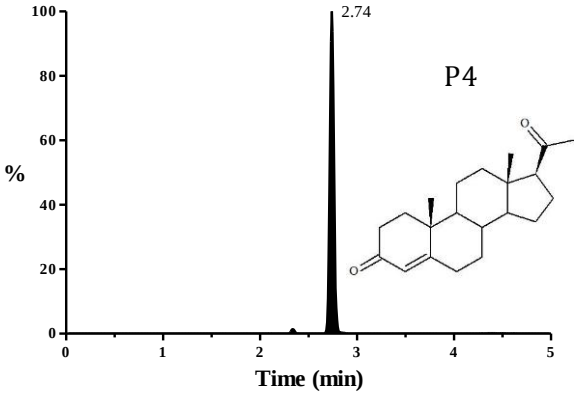
Table 5.5 UHPLC-MS/MS parameters for the separation of fourteen steroids. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.

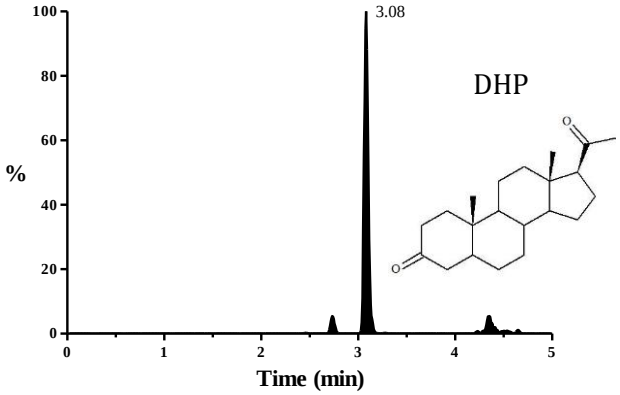
Steroid metabolite	RT (min)	Parent ion $[M+H]^+$	CV	Daughter ion[a] $[M+H]^+$	CE	Daughter ion[b] $[M+H]^+$	CE	Daughter ion[c] $[M+H]^+$	CE
P4	2.74	315.2	30	96.9	15	297.2	15	-	-
DHP	3.08	317	30	95	30	105.2	30	175.2	20
17OHP4	2.10	331.2	26	97	15	108.9	15	-	-
Pdione	2.35	333.4	25	159	20	255	15	-	-
DOF	1.54	347.1	25	121	20	269.2	20	-	-
11OHPdione	1.63	349.2	25	253	30	-	-	-	-
DOE	1.68	345.2	25	121.1	20	162.8	25	-	-
11KPdione	1.74	347.4	25	147	20	269	20	329	15
T	1.94	289.2	30	97.2	22	109	22	-	-
DHT	2.17	291.2	25	255	15	273	20	-	-
D2-T	1.94	291	30	99.1	20	111.25	20	-	-
D7-A4	2.04	294.3	25	100	25	113	25	-	-
D7-11OHA4	1.57	310.2	25	99.8	30	147.2	25	-	-
D9-P4	2.74	324.2	30	100	20	113	25	-	-

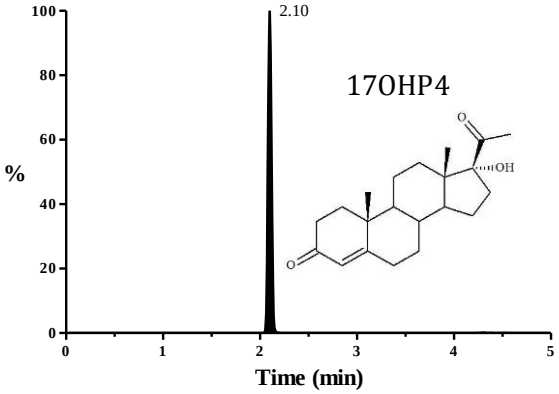
[a]Mass transition of the daughter ion used as quantifier

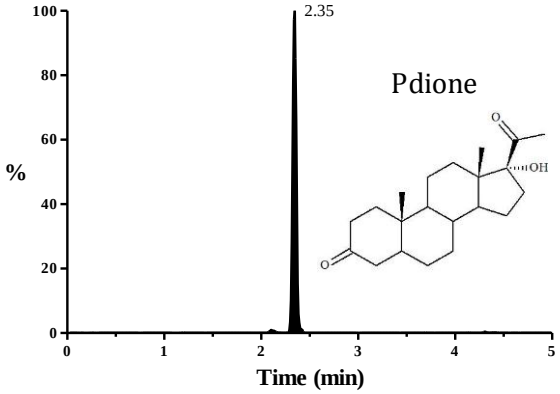
[b,c]Mass transition of the daughter ion used as qualifier

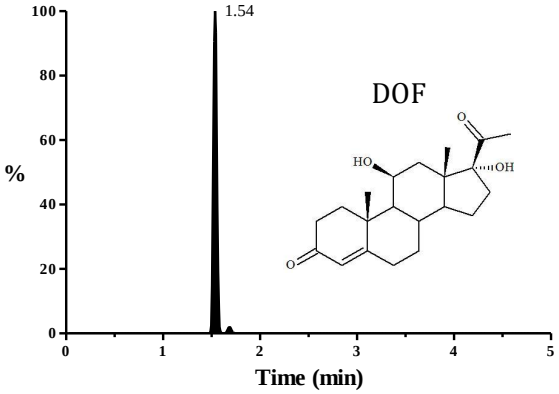
A UHPLC-MS/MS method was established using the parameters in Table 5.5 that could separate fourteen closely related steroid metabolites in a single chromatographic step in 5 minutes. Efficient separation was achieved without sample clean-up or derivatisation illustrated in Figure 5.3.

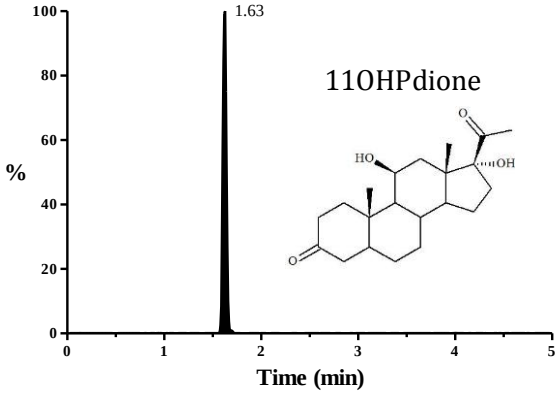


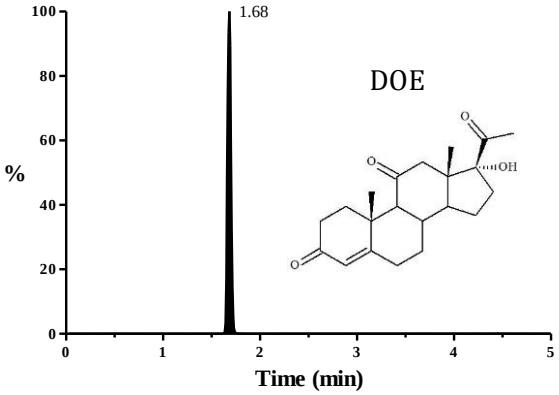


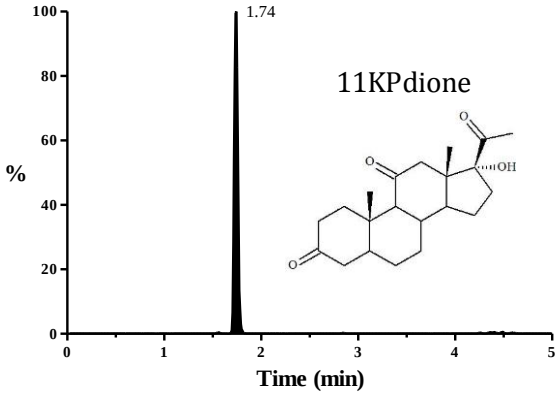












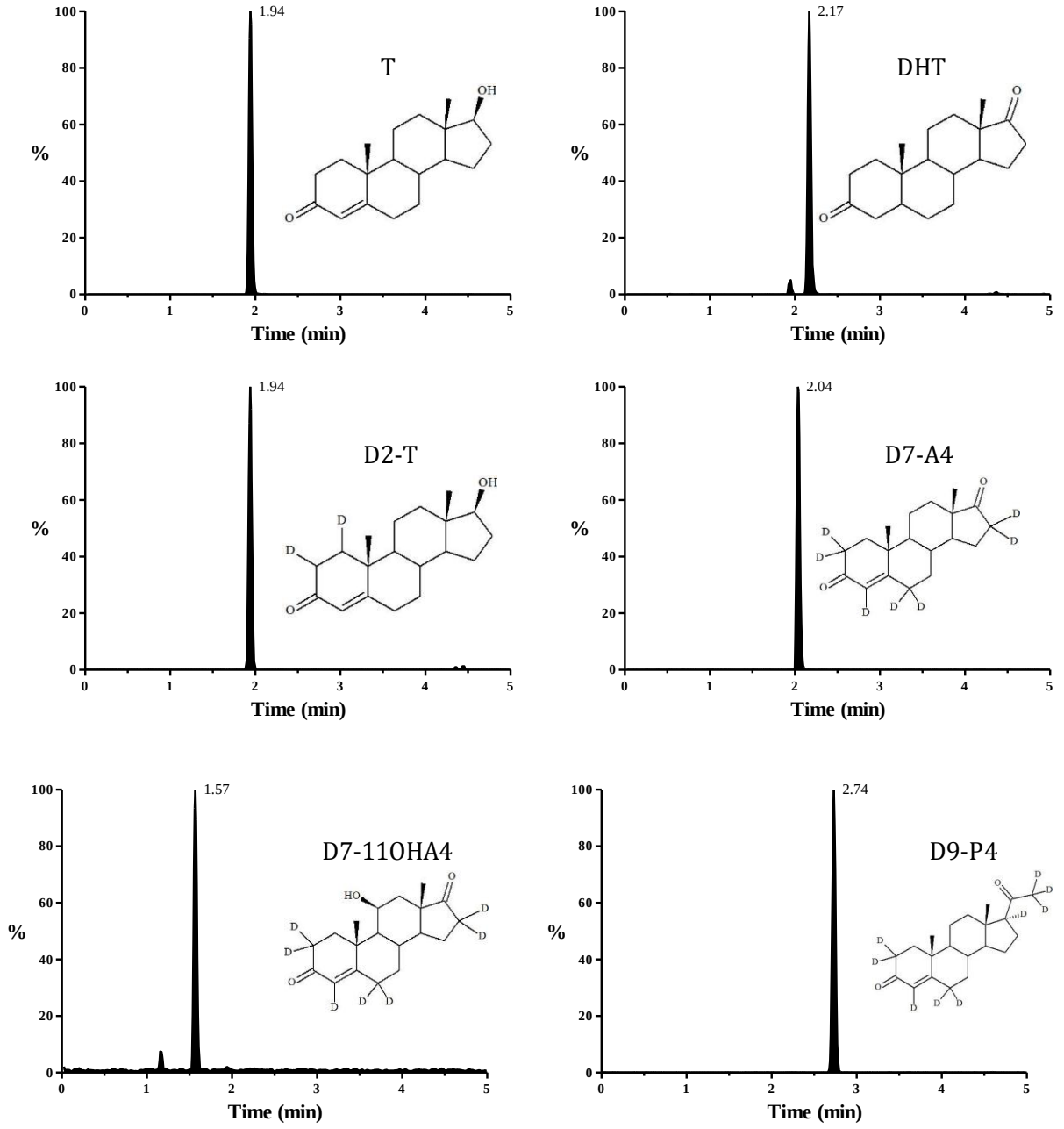


Figure 5.3 UHPLC-MS/MS chromatographic separation of fourteen steroids. The structure and retention time of steroids (shaded peak) are indicated on the chromatograms. Chromatograms were generated using MRM mode (5 µL injection volume of the standard 2.5 ng/µL). Peaks were generated from the highest product in conversion assays for steroids without available standards.

Figure 5.4 depicts the overlaid chromatograms of the efficient chromatographic separation of the steroid metabolites achieved in a single 5 minute run. Internal standards are excluded. Retention times for internal standards were as follows: D2-T, 1.94 minutes; D7-A4, 2.04 minutes; D7-11OHA4, 1.57 minutes; and D9-P4 at 2.74 minutes.

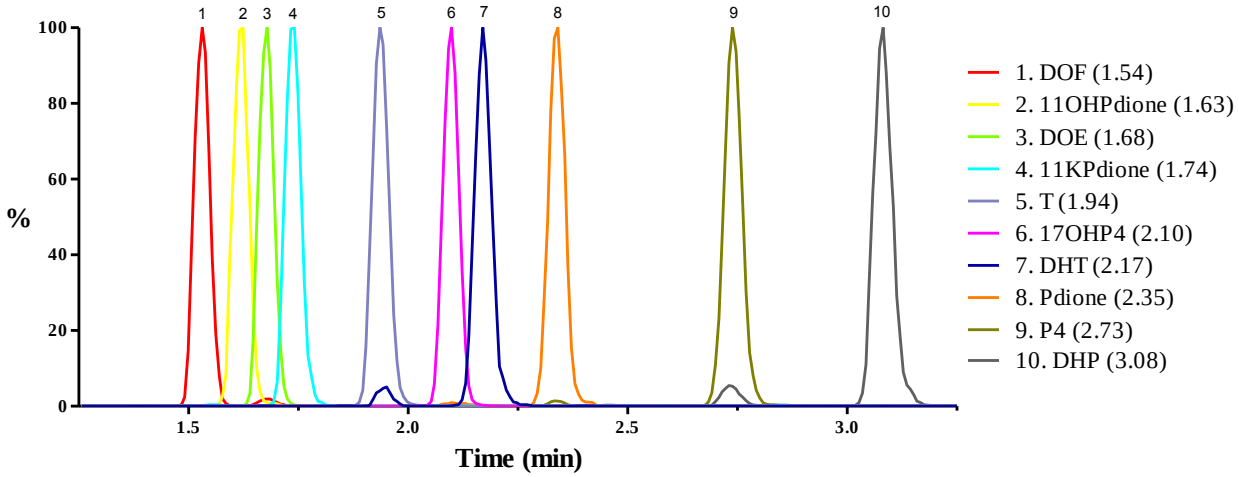


Figure 5.4 Overlaid UHPLC-MS/MS chromatogram of SRD5A substrates and products in MRM mode.

Using the method developed on the UHPLC-MS/MS, the catalytic conversion of P4, 17OHP4, DOF and DOE by the SRD5A isozymes were subsequently investigated.

5.2.2.3 The conversion of C_{21} steroids by SRD5A in HEK293 cells

The metabolism of P4 in the backdoor pathway has been demonstrated (Auchus, 2004) and although the conversion of P4 by SRD5A1 is well-established (Frederiksen & Wilson, 1971), time course data regarding the metabolism by both SRD5A1 and SRD5A2 has not been reported. P4 (5 µM) conversion was thus investigated in HEK293 cells expressing either SRD5A1 or SRD5A2 over the course of 12 hours. SRD5A1 shows a higher catalytic activity towards P4 than SRD5A2 with both enzymes yielding full conversion to DHP after 12 hours (Figure 5.5). SRD5A1 catalysed 50% conversion of P4 within the first hour, while 50% P4 was only converted by SRD5A2 at 3 hours. The data shows that the initial reaction rate of SRD5A1 towards P5, at 5 µM, is markedly faster than that achieved by SRD5A2.

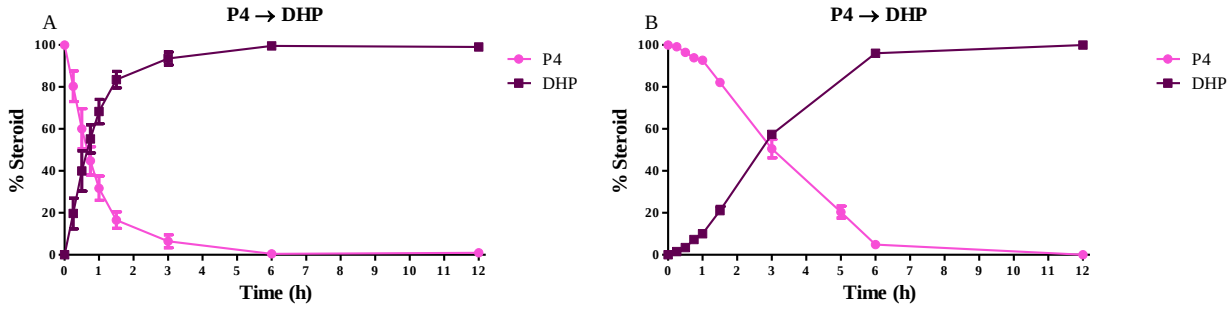


Figure 5.5 Metabolism of 5 µM P4 by SRD5A1 (A) and SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM.

Similarly, 17OHP4 metabolism in the backdoor pathway has been established (Auchus, 2004) and it was already shown in 1971 that SRD5A in rat prostate nuclear cell preparations exhibit a greater catalytic

activity towards 17OHP4 and in comparison, a higher Km towards T and A4 (±2-fold and ±4-fold, respectively). V_{max} values were however not reported (Frederiksen & Wilson, 1971). The metabolism of 17OHP4 over time by these isozymes has also not been reported. The catalytic conversion of 5 µM 17OHP4 was therefore investigated over 12 hours in HEK293 cells transiently transfected with SRD5A1 or SRD5A2. Full conversion was observed with both isoforms at 6 hours (Figure 5.6), comparable to the conversion of P4. Although the conversion of 17OHP4 by the two isozymes appeared to be similar, a marginally higher catalytic conversion is initially observed with SRD5A1, showing 50% conversion at 2 hours and SRD5A2 only at 2.5 hours.

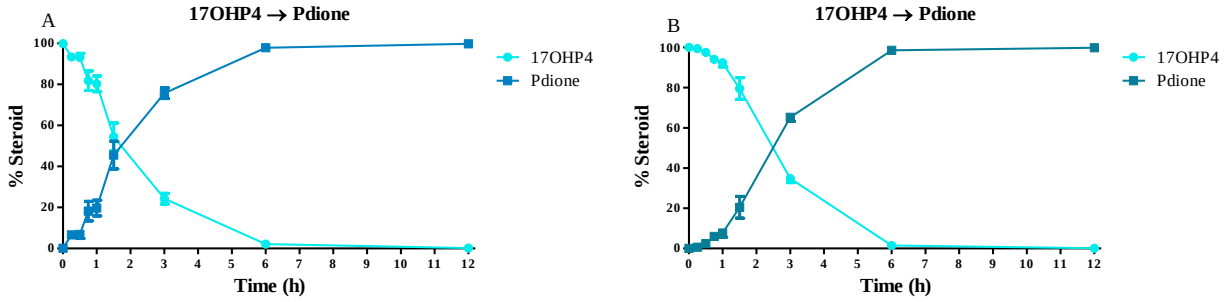


Figure 5.6 Metabolism of 5 µM 17OHP4 by SRD5A1 (A) and SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM.

Even though DOF has been identified in patient serum samples in the previous century, conversion by the enzymes in the backdoor pathway has not, to our knowledge, been investigated. As such, the metabolism of DOF (5µM) by SRD5A1 and SRD5A2 was investigated in transiently transfected HEK293 cells over 12 hours, and yielded the novel steroid metabolite, 11OHPdione. Greater catalytic activity towards DOF was observed with SRD5A2 (Figure 5.7). Full conversion of DOF catalysed by SRD5A1 was not obtained, even after 24 hours (data not shown), with 50% conversion detected only at 10 hours. On the other hand 50% conversion with SRD5A2 was detected at 4 hours and full conversion after 12 hours.

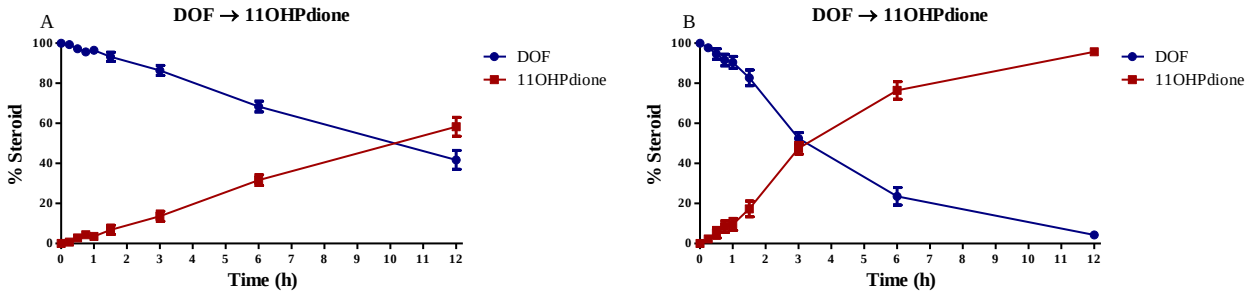


Figure 5.7 Metabolism of 5 µM DOF by SRD5A1 (A) or SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM.

No data regarding the metabolism of DOE by the enzymes in the backdoor pathway has to date been reported. Data demonstrate for the first time the catalytic activity of SRD5A1 and SRD5A2 towards DOE,

yielding the novel steroid metabolite 11KPdione. SRD5A2 showed higher catalytic activity towards DOE than SRD5A1 (Figure 5.8). SRD5A1 catalyse the conversion of DOF, yielding 50% conversion at 8 hours. Full conversion of DOE to 11KPdione was not observed with SRD5A1, even after 24 hours (data not shown). Catalytic conversion of DOE by SRD5A2 showed efficient conversion, yielding 50% conversion after 3 hours and full conversion by 12 hours. Conversion of DOE, catalysed by SRD5A2 is clearly more efficient than conversion catalysed by SRD5A1.

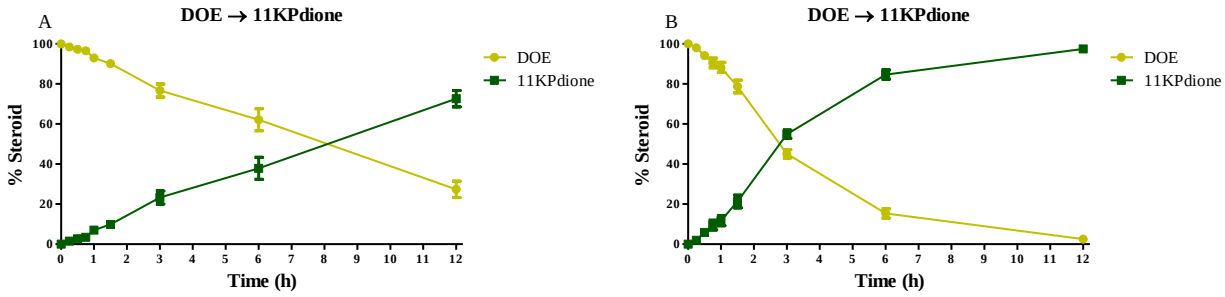


Figure 5.8 Metabolism of 5 µM DOE by SRD5A1 (A) or SRD5A2 (B) in HEK293 cells. Data represent two independent experiments, performed in triplicate, expressed as the mean ±SEM.

Although the data presented were not normalised to protein levels, effective conversion of T by both isozymes was obtained (Figure 5.11), as well as similar trends being obtained in the two independent experiments, enabled the comparative analysis of the data obtained in the conversion assays catalysed by the two isoforms. It is evident that the C11-oxy steroids are not metabolised as readily by SRD5A1 as P4 and 17OHP4 (Figure 59). In addition, the data showed that DOF and DOE are metabolised more efficiently by SRD5A2 than by the type 1 isoform (Figure 5.10). With the exception of the metabolism of DOF and DOE catalysed by SRD5A1, all other steroid substrates were fully converted with the SRD5A isozymes after 12 hours.

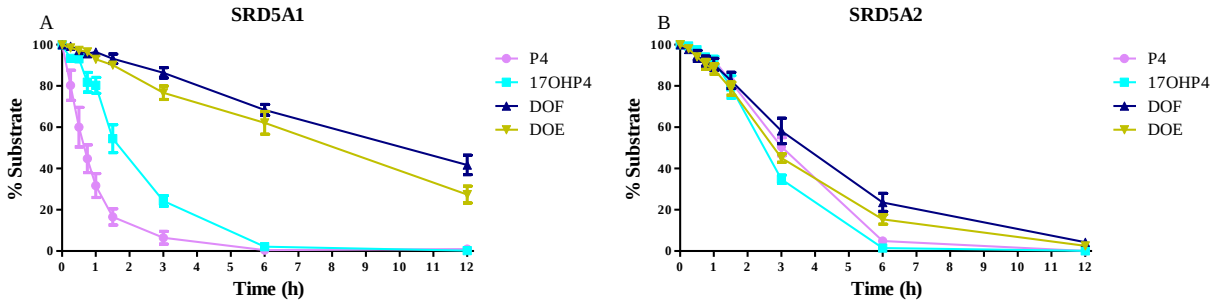


Figure 5.9 Comparison of the catalytic activity of SRD5A1 (A) and SRD5A2 (B) with P4, 17OHP4, DOF and DOE in HEK293 cells. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM.

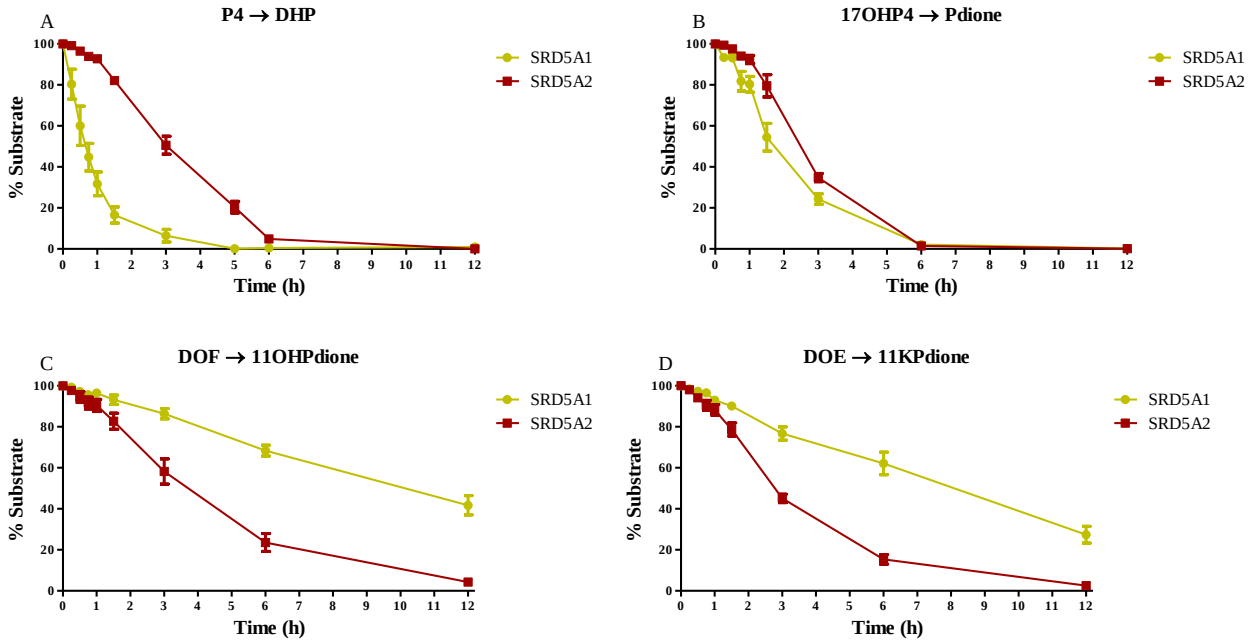


Figure 5.10 Comparison of the catalytic activity of SRD5A1 and SRD5A2 towards P4 (A), 17OHP4 (B), DOF (C) and DOE (D) in HEK293 cells. Results represent two independent experiments performed in triplicate illustrated as the mean ±SEM.

To ensure effective transfection conditions, T (1 µM) was assayed as positive control for the conversions by the SRD5A isozymes (Figure 5.11). Full conversion is observed with both SRD5A1 and SRD5A2 after 12 hours, consistent with previously published data (A. C. Swart et al., 2013).

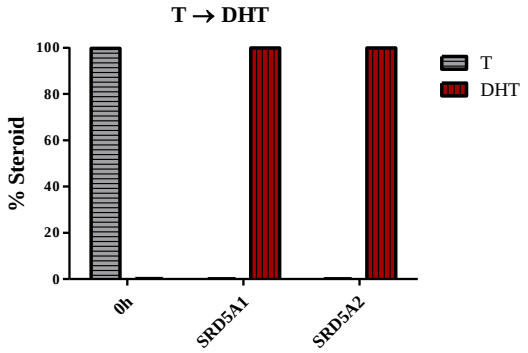


Figure 5.11 Metabolism of 1 µM T by the SRD5A isozymes in HEK293 cells after 12 hours. Results represent two independent experiments, performed in triplicate represented as the mean ±SEM. 0h, sample aliquot collected at start of the assay.

To ensure that the 5α-reduction demonstrated above was due to the transfected plasmid DNA and not the expression of endogenous enzymes, untransfected cells and cells transfected with the pCIneo vector, without insert cDNA, were used as negative control. No substrate conversion was observed in 12 hours (Figure 5.12).

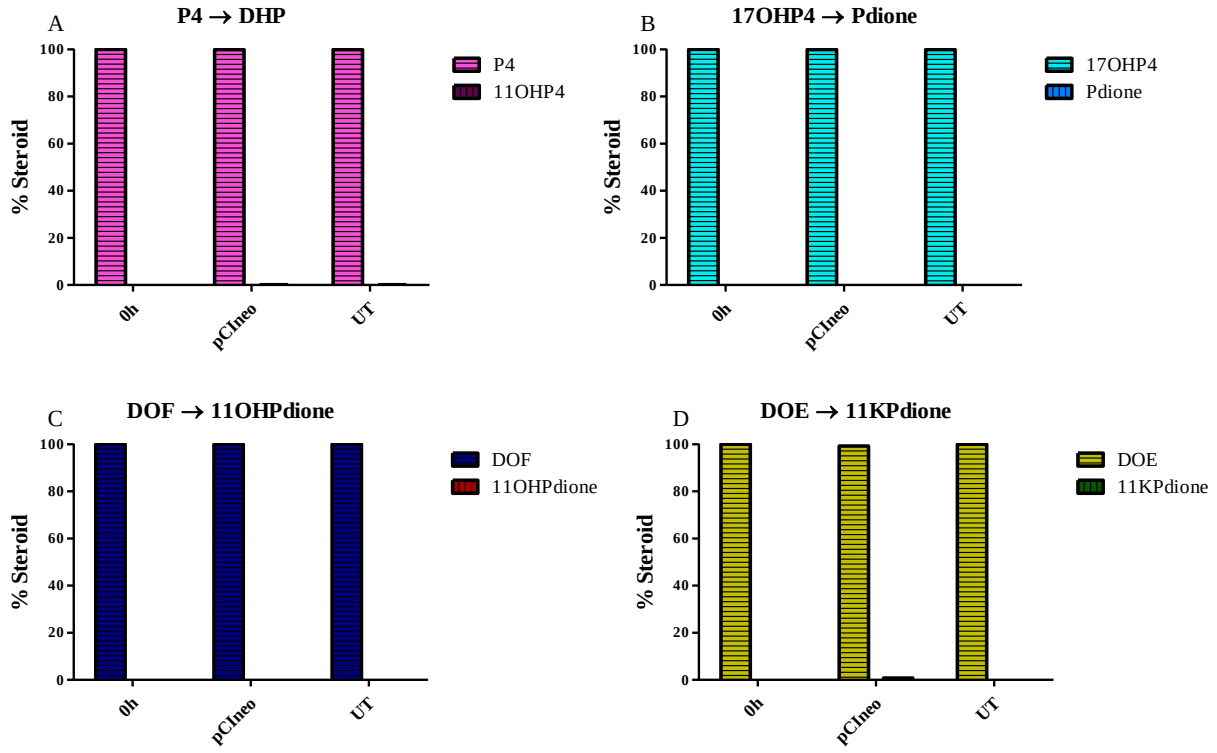


Figure 5.12 Metabolism of 5 µM of P4 (A), 17OHP4 (B), DOF (C) and DOE (D) in HEK293 cells after 12 hours. Results represent two independent experiments performed in triplicate, illustrated as the mean ±SEM. 0h, sample aliquot collected at start of the assay; pCIneo, HEK293 cells transfected with vector without cDNA (negative control); UT, untransfected HEK293, (control, endogenous enzyme expression).

Taken together, the SRD5A subtypes shows clear activity towards DOF and DOE, albeit at different reaction rates, allowing the first step in the backdoor pathway to take place, yielding the novel steroids 11OHPdione and 11KPdione.

5.2.3 The conversion of 5α-reduced C_{21} steroids, 11OHPdione and 11KPdione by AKR1C2 in HEK293 cells

5.2.3.1 Accurate mass determination of 11OHPdiol and 11KPdiol

The confirmation of the catalytic conversions of DOF and DOE by the SRD5A isozymes prompted further investigations into the conversion of the 5α-reduced products, 11OHPdione and 11KPdione, by AKR1C2. The conversion of 11OHPdione and 11KPdione (the SRD5A reduced products of DOF and DOE) was investigated in HEK293 cells transiently transfected with pcDNA3/hAKR1C2. The accurate mass of 11OHPdiol and 11KPdiol was determined using Q-tof-MS (Figure 5.13).

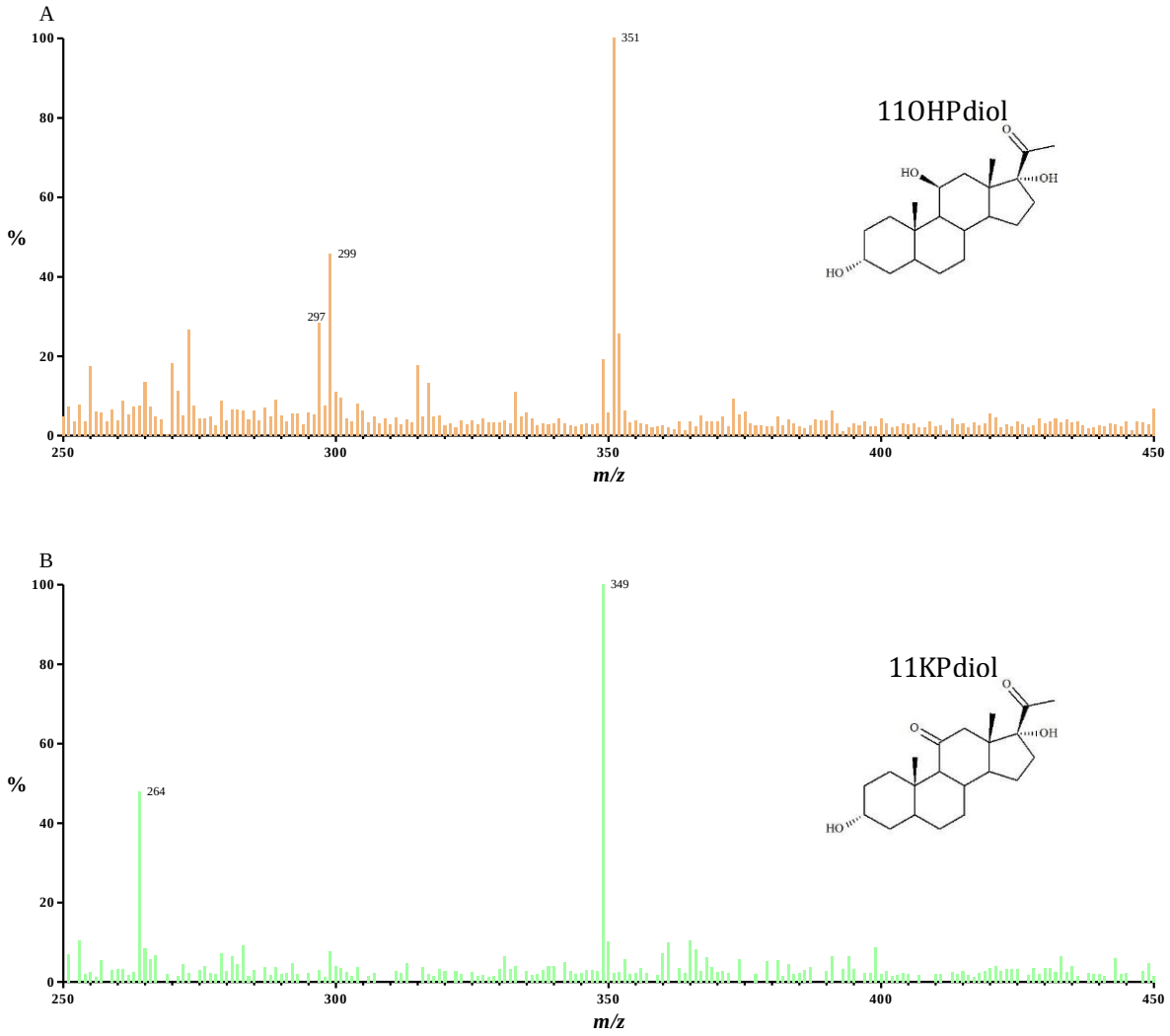


Figure 5.13 MS spectra of (A) 11OHPdiol ($[M+H]^+$ *m/z* 351.2) and (B) 11KPdiol ($[M+H]^+$ *m/z* 349.2). Acquired mass range was scanned across *m/z* 150-600.

Once the 11OHPdiol and 11KPdiol products were established by accurate mass, further fragmentation was carried out from the selected parent ions in a coupled collision cell. The data obtained from the Q-tof-MS/MS analyses are summarised in Table 5.6.

Table 5.6 Accurate mass determination of 11OHPdiol and 11KPdiol. Accurate masses (observed and calculated), formulae and fragment ions are depicted.

Compound	Observed accurate mass $[M+H]^+$	Calculated mass	Formula	Fragments $[M+H]^+$
11OHPdiol	351.1991	351.2535	$C_{21}H_{34}O_4$	253, 271
11KPdiol	349.2303	349.2379	$C_{21}H_{32}O_4$	107, 147, 271

The identification of 11OHPdiol an 11KPdiol and fragmentation data obtained from the Q-tof-MS/MS allowed further investigation on the UHPLC-MS/MS in MRM mode.

5.2.3.2 UHPLC-MS/MS analyses of steroid metabolites

Using the accurate mass and fragments obtained from the Q-tof-MS/MS, a method for the separation of the steroid metabolite of interest for the AKR1C2 conversions was developed (Table 5.7).

Table 5.7 UHPLC-MS/MS parameters for the separation of steroid metabolites for AKR1C2 conversions. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.

Steroid metabolite	RT (min)	Precursor ion $[M+H]^+$	CV	Product ion[a] $[M+H]^+$	CE	Product ion[b] $[M+H]^+$	CE	Product ion[c] $[M+H]^+$	CE
T	2.19	289.2	30	97.2	22	109	22	-	-
DHT	2.44	291.2	25	255	15	273	20	-	-
Pdione	2.66	333.4	25	159	20	255	15	-	-
Pdiol	2.80	317	20	111	25	299	15	175.2	20
DOF	1.68	347.1	25	121	20	269.2	20	-	-
11OHPdione	1.79	349.2	25	253	30	-	-	-	-
11OHPdiol	1.55	351.2	30	253	20	271	15	-	-
DOE	1.88	345.2	25	121.1	20	162.8	25	-	-
11KPdione	1.96	347.4	25	147	20	269	20	329	15
11KPdiol	1.65	349.2	30	107	20	147	20	271	20
D2-T	2.19	291	30	99.1	20	111.25	20	-	-
D7-A4	2.30	294.3	25	100	25	113	25	-	-
D7-11OHA4	1.71	310.2	25	99.8	30	147.2	25	-	-
D9-P4	3.10	324.2	30	100	20	113	25	-	-

[a]Mass transition of the daughter ion used as quantifier

[b,c]Mass transition of the daughter ion used as qualifier

Since no standards were commercially available for 11OHPdione and 11KPdione, these steroid metabolites were produced in HEK293 cells expressing SRD5A2, the isoform which was shown to convert 11OHPdione and 11KPdione fully by 12 hours. After 12 hours the steroid products were carried over to HEK293 cells transiently transfected with AKR1C2. Even though all metabolites could be separated, low sensitivity and high crosstalk between the different steroid metabolites hampered analyses. Figure 5.14 illustrates the separation of the DOF and DOE metabolites respectively.

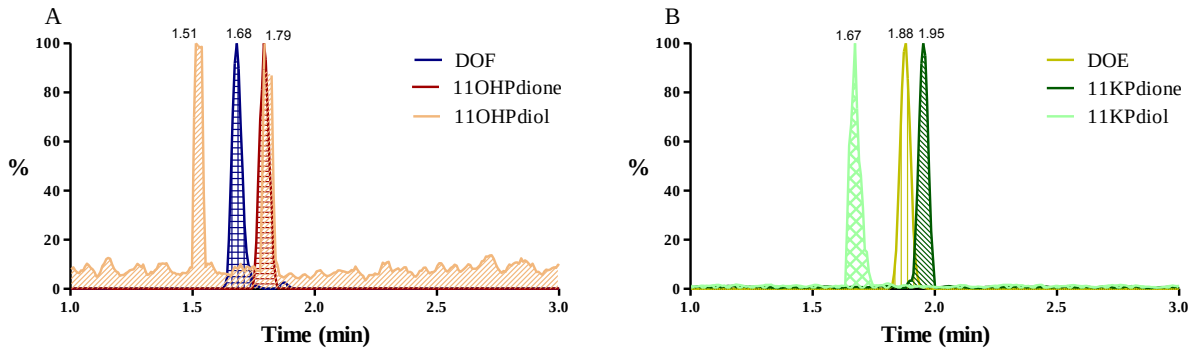


Figure 5.14 UHPLC-MS/MS analyses of DOF (A) and DOE (B) metabolites. Retention times are noted above each peak.

Analyses of the catalytic conversions were hindered by the low intensity observed with the 11OHPdiol and 11KPdiol products. Accurate mass determinations and preliminary studies indicated the production of 11OHPdione and 11KPdione and the subsequent metabolism of these intermediary steroids by AKR1C2 leading to the production of 11OHPdiol and 11KPdiol, confirming the second step in the backdoor pathway.

5.2.4 DOF metabolism in cell models

Having established that DOF is converted by both SRD5A and AKR1C2 in transfected cells, the conversion of DOF was investigated in prostate cancer cell models to establish conversion in a cell system expressing a cohort of steroidogenic enzymes.

5.2.4.1 UPC^2-MS/MS analyses of C11-oxy C_{21} steroids

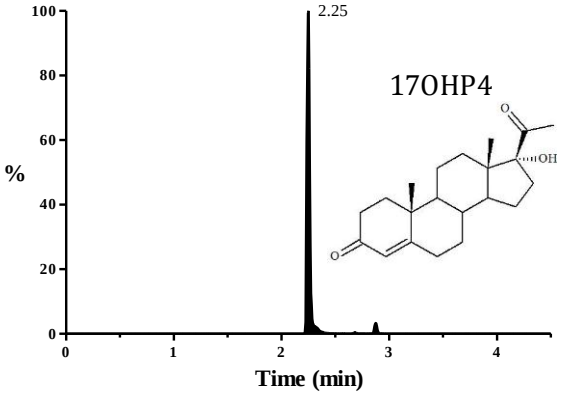
UPC^2-MS/MS showed improved sensitivity and accuracy in comparison to the UHPLC-MS/MS method. A method was therefore developed to investigate DOF metabolism in different cell models (Table 5.8), and chromatographic separation of the steroid metabolites are depicted in Figure 5.15.

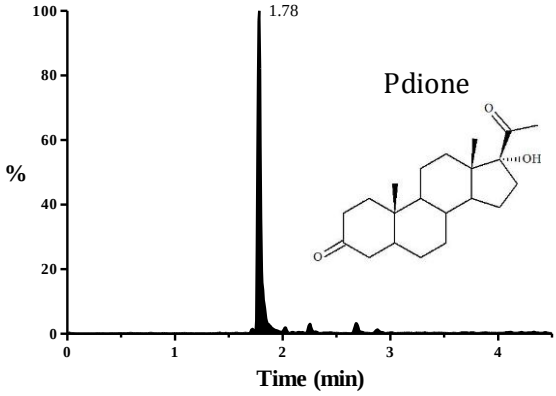
Table 5.8 UPC^2-MS/MS parameters for the separation of steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.

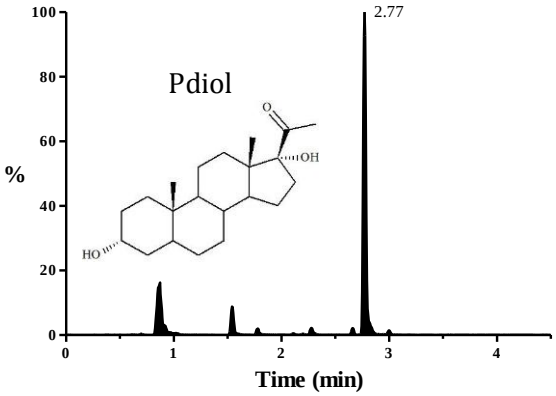
Steroid metabolite	RT (min)	Parent ion $[M+H]^+$	CV	Daughter ion[a] $[M+H]^+$	CE	Daughter ion[b] $[M+H]^+$	CE	Daughter ion[c] $[M+H]^+$	CE
DHT	1.85	291.2	25	255	15	273	20	-	-
17OHP4	2.25	333.1	26	97	22	109	28	-	-
Pdione	1.78	333.4	20	137	25	159	25		-
Pdiol	2.77	317	20	111	25	199	15	175.2	20
AST	1.86	291.3	18	105.3	30	273.2	8	-	-
DOF	3.21	347.1	20	121	25	269.2	15	-	-
11OHPdione	2.84	349.2	25	253	30	-	-	-	-
11OHAST	2.90	289	15	213	15	271	15	-	-
DOE	2.64	345.2	25	121.1	20	162.8	25	-	-
11KPdione	2.30	347.4	25	147	20	269	20	329.2	15
11KAST	1.68	305	30	147.2	30	173.1	30	-	-
11KPdiol	2.96	349.2	30	107	20	147	20	271	20
D2-T	2.40	291	30	99.1	20	111.25	20	-	-
D7-A4	1.66	294.3	25	100	25	113	25	-	-
D7-11OHA4	2.54	310.2	25	99.8	30	147.2	25	-	-
D9-P4	1.54	324.2	30	100	20	113	25	-	-

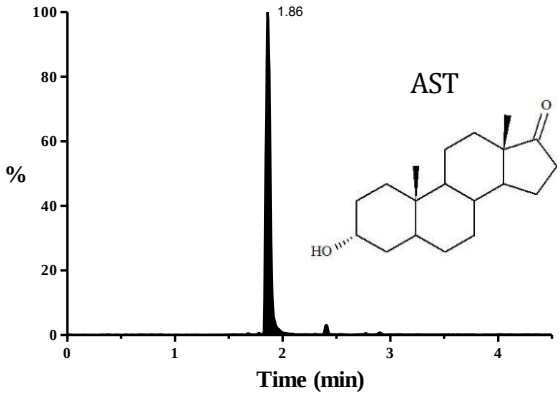
[a]Mass transition of the daughter ion used as quantifier

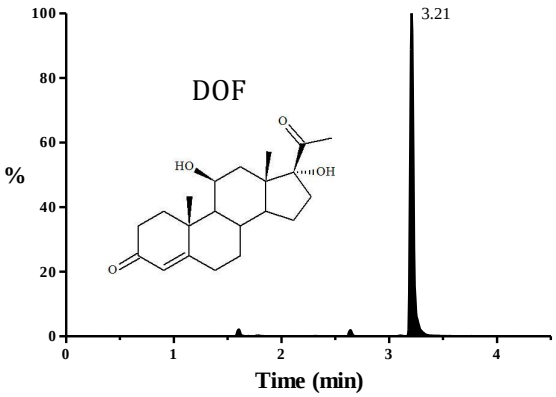
[b,c]Mass transition of the daughter ion used as qualifier

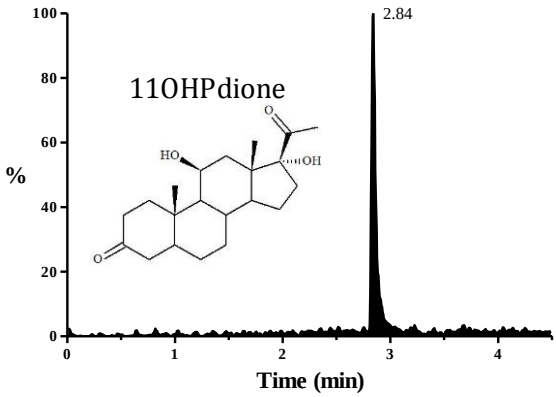


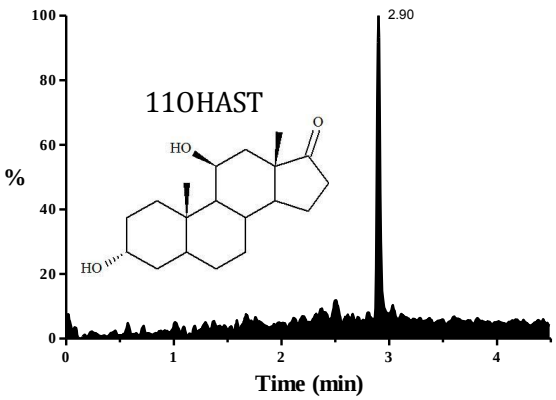


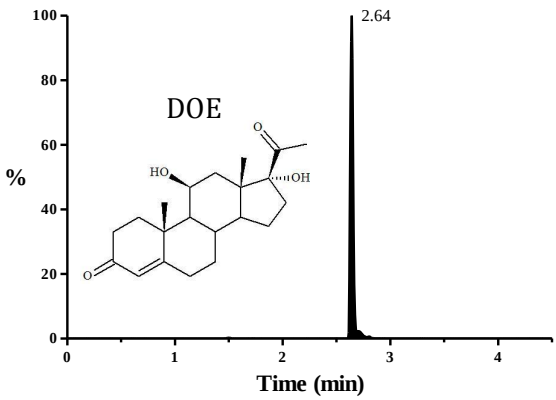












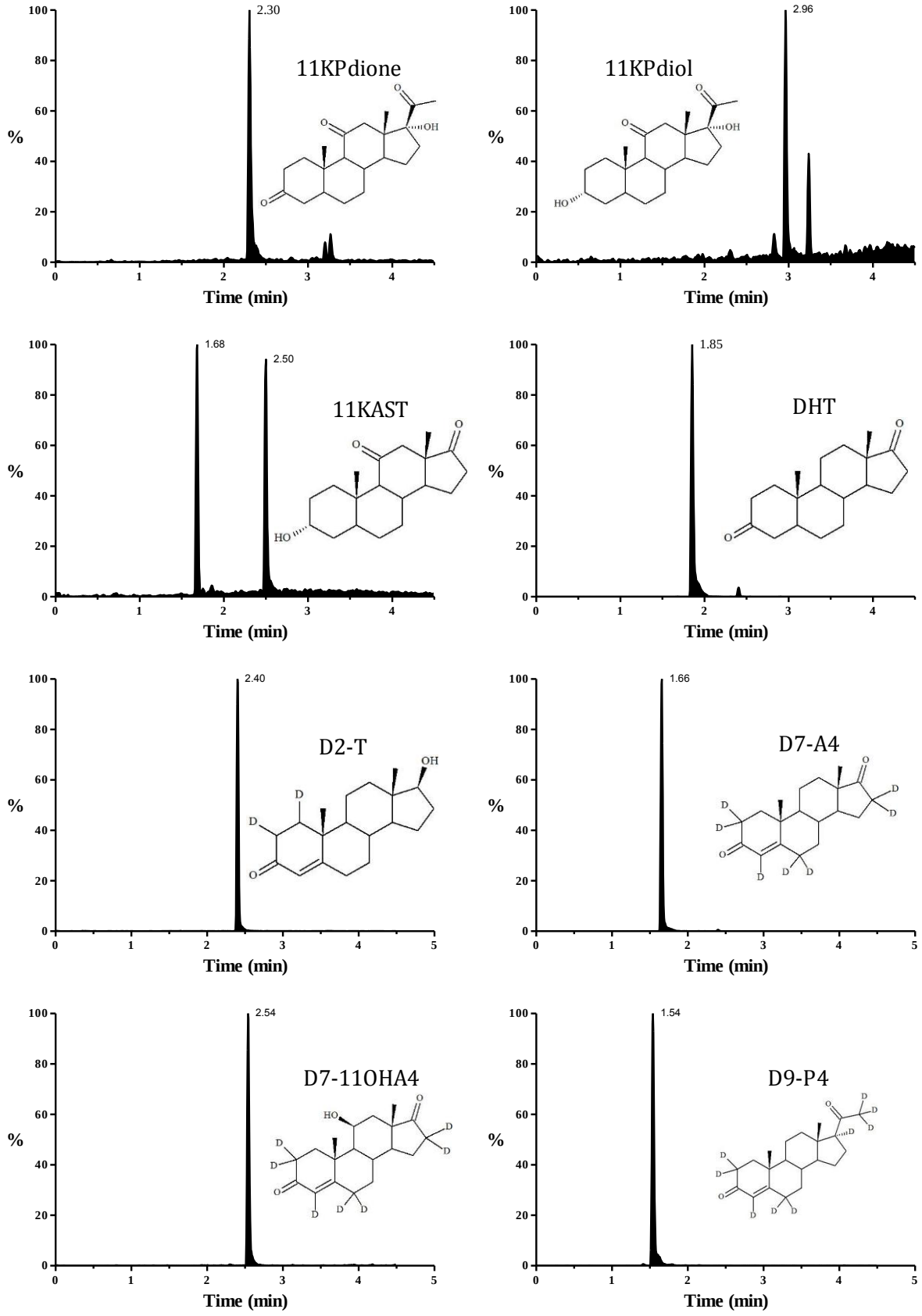


Figure 5.15 UPC^2-MS/MS chromatographic separation of 16 steroids. The structure and retention times of steroids (shaded area) are indicated on the chromatograms. Chromatographic profiles were generated using MRM mode (2 µL injection of standard, 1 µg/mL).

The UPC^2-MS/MS method allowed investigations into different cell models, sensitive at low steroid concentrations.

5.2.4.2 DOF conversion in PNT2, PC3 and LNCaP cells

Using the UPC^2-MS/MS method, DOF metabolism in PC3, PNT2 and LNCaP cells was analysed and quantified. DOF conversion was most prominent in LNCaP cells and yielded significant levels of DOE, indicating the presence of 11βHSD2 activity (Figure 5.17).

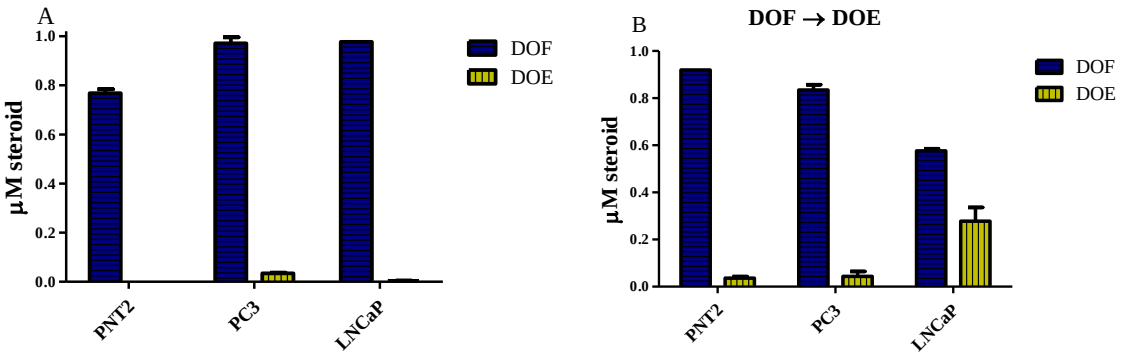


Figure 5.16 The metabolism of 1 µM DOF in PC3, PNT2 and LNCaP cell models. Steroid concentrations at 0 hour (A) and at 24 hours (B).

The metabolism of DOF in LNCaP cells showed the highest downstream conversion of the three cell models investigated. More than 40% DOF was metabolised after 24 hours, yielding DOE (±0.3 µM), 11OHAST (<0.1 µM) and 11KAST (±0.15 µM) (Figure 5.17). 11OHPdione was detected however, the response was low and could not be quantified since a standard for this product is not commercially available. Results obtained from LNCaP analyses depict the total steroids (glucuronidated and free) observed after the deconjugation of bound steroids. Metabolism in PNT2 cells yielded marginal levels of DOE and 11OHAST, but no 11KAST. Similarly, negligible conversion was observed in PC3 cells, yielding less than 0.1 µM DOE, 11OHAST and 11KAST. The 11OHPdione levels in PNT2 and PC3 cells were lower than that of LNCaP cells.

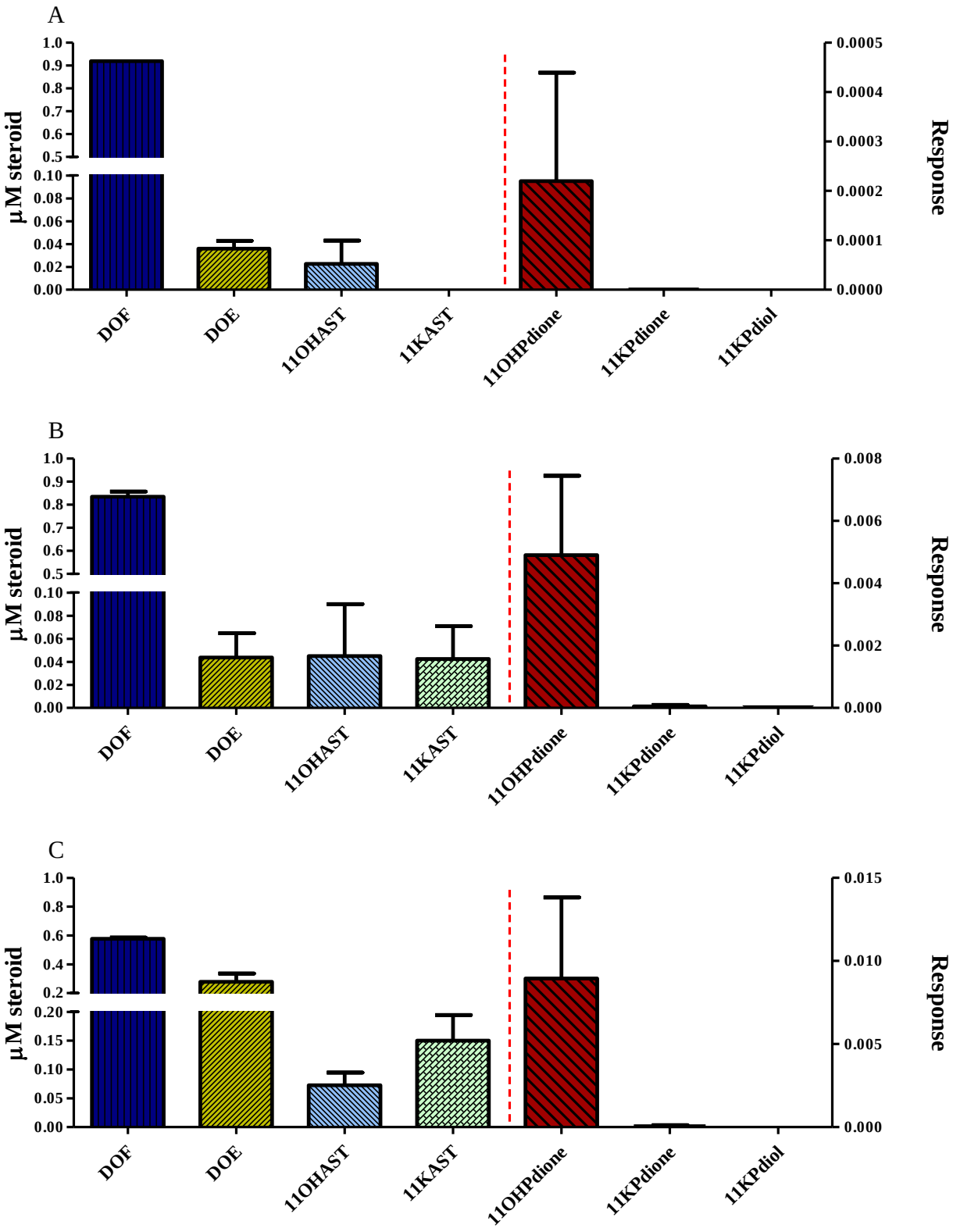


Figure 5.17 Metabolism of 1 µM DOF in PNT2 (A), PC3 (B) and LNCaP (C) cells after 24 hours. Results represent triplicate values as mean ±SEM. Results are depicted in µM concentrations and as response in the case of steroids for which no standards were available.

17OHP4 (1 µM) metabolism was used as control, since downstream conversion of this steroid in the backdoor pathway has been demonstrated. Limited conversion of 17OHP4 was observed in the three cell models, and higher conversion of DOF was observed compared to that of 17OHP4.

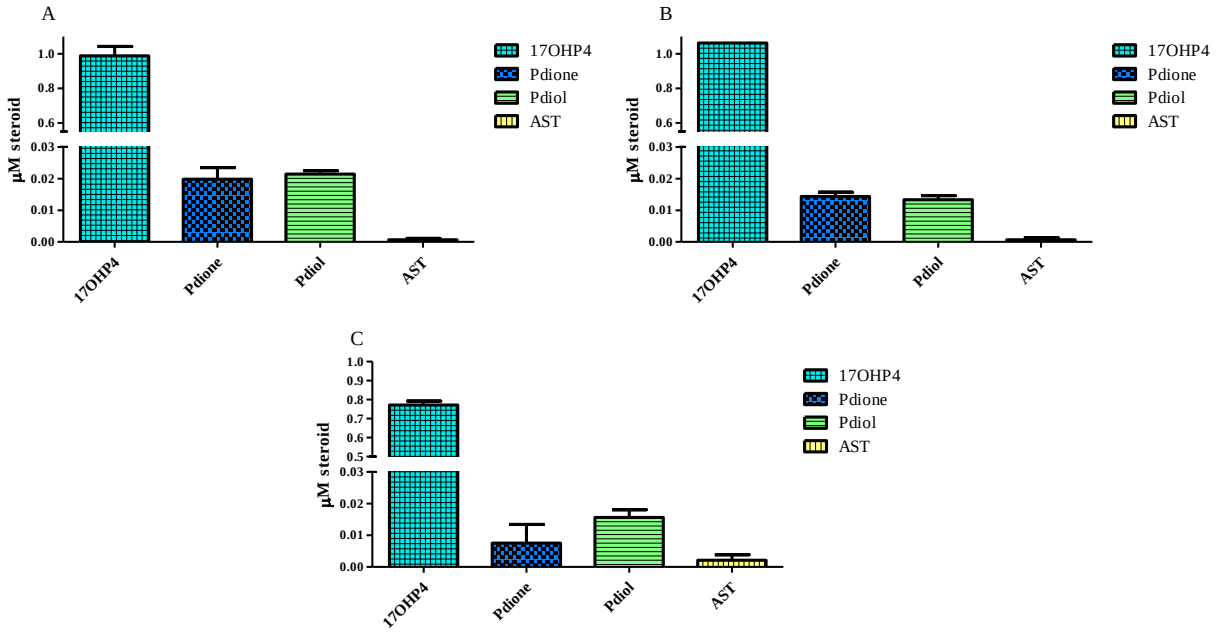


Figure 5.18 Metabolism 1 µM 17OHP4 in PNT2 (A), PC3 (B) and LNCaP (C) cells after 24 hours. Results represent triplicate values as mean ±SEM.

From the data presented in this chapter, it is clear that DOF and DOE are metabolised by the enzymes in the backdoor pathway. Since this was a pilot study, novel methods developed on the UHPLC-MS/MS and UPC^2-MS/MS systems needs further full validation, not included in the scope of this study.

5.3 Discussion

The adrenal is an important source of androgen precursors, with the downstream conversion of these steroids playing a central role in normal and disease physiology. The recently reported 11OHA4 pathway has added a level of complexity to the production of androgens from adrenal origin, highlighting the importance of adrenal androgen precursors in androgen-dependant diseases. In addition, the backdoor pathway has been put forward as an important biosynthesis pathway in the production of DHT, using 17OHP4 as precursor, bypassing the intermediates in the conventional DHT biosynthesis pathway. It has been reported that androgens produced via the backdoor pathway contribute significantly to excess androgens characteristic of 21OHD (Kamrath, Hochberg, et al., 2012). In the light of studies reporting elevated levels of 11OHA4 pathway metabolites in serum samples of 21OHD patients (A. F. Turcu et al., 2016) and taking into account the 11β-hydroxylation of 17OHP4, it is likely that the products, DOF and DOE, contribute to the C11-oxy androgen pool of steroids via the backdoor pathway.

In this study we investigated the catalytic activity of human SRD5A1 and SRD5A2 towards DOF and DOE in comparison to P4 and 17OHP4. The 5α-reductase enzymes are central in the production of potent androgens, present in all the aforementioned pathways involved in the production of potent androgens (Chapter 3). Specifically, within the backdoor pathway, SRD5A enzymes catalyses the first step towards androgen biosynthesis. The data presented in this chapter clearly demonstrate that DOF and DOE are suitable substrates for the 5α-reduction, yielding the respective novel steroid metabolites, 11OHPdione and 11KPdione. In 21OHD the production of DOF and DOE will yield substrates to be metabolised by SRD5A isozymes, specifically SRD5A2. The metabolism of DOF and DOE by the type 2 isozyme was comparable to the metabolism of P4 and 17OHP4, known substrates in the backdoor pathway. As 21OHD is characterised by elevated 17OHP4 and DOF levels (A. F. Turcu et al., 2015), our data suggest that the backdoor pathway may be even more prominent in the production of potent androgens, with DOF and DOE being reduced by SRD5A, potentially leading to the production of 11OHDHT and 11KDHT.

In the next intermediary step - the metabolism of 11OHPdione and 11KPdione by AKR1C2, the novel steroid metabolites 11OHPdiol and 11KPdiol were identified by accurate mass determination. This is a crucial reduction step in the backdoor pathway and a necessity for the production of substrates for the lyase activity of CYP17A1 (Gupta et al., 2003). The production of 11OHPdiol and 11KPdiol, potentially high affinity substrates for CYP17A1, would yield 11OHAST and 11KAST, which may contribute to the circulating pool of steroid metabolites. 11OHAST has been detected as a primary metabolite of 11OHA4 and present in high levels in urine of children with 21OHD (Kamrath et al., 2017), however the production of 11OHAST via the metabolism of DOF in the backdoor pathway can contribute to the circulatory levels of 11OHAST detected in the aforementioned study. It is therefore crucial that studies investigating serum and urinary profiles of 21OHD include C11-oxy steroid metabolites presented in both the 11OHA4 and backdoor pathway, since the production of these steroids can no longer only be ascribed to the downstream metabolism of 11OHA4.

This is supported by studies carried out in prostate cancer cell models. DOF metabolism was most prominent in LNCaP cells, a CRPC model, that express high levels of AKR1C2 as well as SRD5A1 (Luu-The et al., 2008), with studies demonstrating that CYP17A1 expression is up-regulated in these cells under certain treatment conditions (Bremmer et al., 2014). The metabolism of DOF yielded both 11OHAST and 11KAST, the CYP17A1 lyase products of 11OHPdiol and 11KPdiol. The intermediary products, with the exception of 11OHPdione, were not detected illustrating that the formed intermediary products are effectively lyased by CYP17A1. We can therefore deduct that the novel C11-oxy C_{21} steroids, 11OHPdiol and 11KPdiol, are substrates for the lyase activity of CYP17A1. This is in agreement with studies that showed 5α-, 3α-reduced steroid metabolites to be high affinity substrates for the lyase activity of CYP17A1 (Gupta et al., 2003). This data furthermore support the production of DOE from DOF by 11βHSD2, since DOF metabolism in LNCaP cells yielded significant levels of DOE after 24 hours. Studies in our group have shown that the 11βHSD enzymes favour the oxidative activity in the 11OHA4 pathway, ultimately

contributing to the production of 11KDHT (Storbeck et al., 2013). A similar trend is observed in LNCaP cells, with levels of 11KAST being 2-fold higher than 11OHAST. Increased 11KAST can also be ascribed to downstream metabolism of DOE, and conversion of 11OHAST to 11KAST due to 11βHSD2 activity. It is furthermore interesting to note, that higher downstream conversion was observed with DOF as substrate compared to 17OHP4, possibly indicative of the low levels of CYP17A1 expression. The 17OHP4 substrates may also be more readily conjugated by UGT2B enzymes, hampering further downstream conversion. The C11-oxy group may hinder conjugation of DOF metabolites.

The identification of 11OHAST and 11KAST from DOF metabolism in LNCaP cells indicate C11-oxy C_{21} steroids produced by the adrenal can contribute to the production of C11-oxy androgens. Studies in our laboratory are currently investigating the conversion of 11OHAST, 11KAST and 11KAdiol by 17βHSD6 (which catalyses the conversion of Adiol to DHT (Mohler et al., 2011)), which would ultimately contribute to the production of 11KDHT. The conversion of 11OHAST to 11KAST and 11KAdiol by 11βHSD2 and 17βHSD has already been reported (A. C. Swart & Storbeck, 2015). The production of 11OHDHT and 11KDHT from DOF and DOE via enzymes in the backdoor pathway is of importance when the AR agonist activities of these steroids are considered. Both 11OHDHT and 11KDHT can activate the AR, with 11OHDHT androgenicity comparable to T and 11KDHT comparable to DHT at 1nM (Storbeck et al., 2013), while at 10nM both 11OHDHT and 11KDHT showed full agonist activity, equipotent with DHT (Bloem et al., 2015). The production of these steroids via the backdoor pathway, with DOF and DOE as initial substrates may therefore indeed contribute to androgen excess associated with 21OHD.

Taken together, the data show that DOF and DOE serve as substrates for the SRD5A isozymes, yielding the novel steroid metabolites 11OHPdione and 11KPdione. The subsequent catalysis by AKR1C2 leads to the biosynthesis of the novel steroid metabolites 11OHPdiol and 11KPdiol. Downstream metabolism of DOF in LNCaP cells yielded 11OHAST and 11KAST, illustrating the lyase activity of CYP17A1 towards 11OHPdiol and 11KPdiol. DOF and DOE may therefore be prominent players in the production of active androgens in 21OHD, a role attributed to the metabolism by enzymes in the backdoor pathway, yielding 11OHDHT and 11KDHT.

CHAPTER 6

CONCLUSION AND FUTURE PRESPECTIVES

The central aim of this study was to investigate the biosynthesis and downstream metabolism of C11-oxy C_{21} steroids, relevant to the 21OHD adrenal disorder. The most characteristic trait of 21OHD is androgen excess which results in a broad spectrum of clinical symptoms. The production of potent androgens is dependent on the biosynthesis of adrenal androgen precursors, especially in females. Potent androgens are produced from adrenal androgen metabolites by several pathways in peripheral target tissue and the interconversion of active and inactive steroid metabolites is dependent on cell-specific enzyme expression. In addition, 21OHD present with increased levels of CYP21A2 substrates, P4 and 17OHP4. The backdoor pathway describes the downstream metabolism of these adrenal metabolites to DHT, implicating these steroids in the production of potent androgens. CYP21A2 is essential in the production of glucocorticoids and mineralocorticoids and since adrenal production of these hormones is diminished, 21OHD patients are treated with synthetic glucocorticoids and mineralocorticoids to correct effects of the loss of CYP21A2, together with anti-androgens to treat the symptoms of androgen excess. Screening for 21OHD is, however, still unsatisfactory and is hampered by false-positive results and biomarker specificity. Disease diagnoses are furthermore often overshadowed by the development of secondary conditions of androgen excess such as PCOS or TART and an increasing number of studies are now identifying these patients as 21OHD patients, albeit having the less severe, late onset form of the disorder (NC 21OHD).

The high levels of P4 and 17OHP4 observed in 21OHD patients present substrates for the CYP11B isozymes and the subsequent biosynthesis of adrenal C11-oxy C_{21} steroids may contribute to the downstream peripheral production of potent androgens implicated in the androgen excess characteristic of 21OHD. The activity of the CYP11B isozymes were until recently associated only with the production of glucocorticoids and mineralocorticoids. Our research group has however established that this is not the case, and showed the C11-oxy metabolites to be precursors for potent androgens, metabolised in a similar manner as A4 and T, adding a level of complexity to androgen biosynthesis and metabolism (Bloem et al., 2015; A. C. Swart & Storbeck, 2015). These data showed that the CYP11B1 isozymes are not only involved in the production of mineralocorticoids and glucocorticoids but are also implicated in the biosynthesis of adrenal androgen precursors. This study therefore investigated the 11β-hydroxylation of P4 and 17OHP4, and the potential production of potent androgens in the downstream metabolism by steroidogenic enzymes. In chapter 4 we clearly demonstrated the 11β-hydroxylation of P4 and 17OHP4 by both CYP11B isozymes, yielding 11OHP4 and DOF. However, the conversion of P4 by CYP11B1 was more rapid than by CYP11B2, comparable to the conversion of A4, while CYP11B1 and CYP11B2 converted 17OHP4 at a comparable rate. Given the zone specific expression of the isozymes, the production of P4 in the zG would thus not specifically favour the biosynthesis of 11OHP4 in this zone.

However, the expression of CYP17A1 in the zF, presenting both P4 and 17OHP4 to CYP11B1 expressed in this zone, would readily lead to the production of 11OHP4 and DOF.

The analyses of a steroid panel of DOF metabolites, catalysed by the enzymes in the backdoor pathway, detected DOE as the main metabolite of DOF metabolism in LNCaP cells. This is indicative of 11βHSD2 activity in LNCaP cells and suggests competition between 11βHSD2 and SRD5A1 for DOF, with 11βHSD2 preferentially converting DOF to DOE. This correlates with studies of C11-oxy C_{19} steroid metabolism in LNCaP cells via the 11OHA4 pathway that yielded mainly the C11-keto derivatives (Bloem et al., 2015). Since enzyme expression levels within these cells differ, the metabolites detected only confirm reaction catalysis, without any indication of individual enzyme substrate preference, which would have to be confirmed in transfected cell model systems heterologously expressing the specific enzymes. In a clinical setting, metabolites from the conventional-, alternative 5α-dione-, 11OHA4- and backdoor pathways would compete as substrates for important downstream enzymes, specifically SRD5A. With this in mind, 11OHAST and 11KAST levels reported in the DOF metabolism were similar to the metabolite levels reported in studies investigating the metabolism of 11OHA4 in the 11OHA4 pathway which also yielded 11OHAST and 11KAST, as well as higher levels of intermediary products. Intermediates were not detected in the metabolism of DOF, which may indicate that the conversion of 11OHPdione and 11KPdione catalysed by AKR1C2 is more efficient than the C11-oxy C_{19} intermediates, with levels being below the limit of detection. In addition the metabolism of DOF was assayed after 24 hours, while the published data report conversions at 72 hours (Bloem et al., 2015). It is interesting to note that while AKR1C2 inactivates C_{19} and C11-oxy C_{19} steroids in the metabolism of androgens, the enzyme has an activating role in the metabolism of C_{21} steroids in the backdoor pathway, yielding suitable substrates for the lyase activity of CYP17A1 in the conversion of C_{21} to C_{19} steroids. It therefore seems that the preference and expression of AKR1C2 and 17βHSD6, catalysing the reductive and oxidative metabolism of Adione and DHT to AST and Adiol, respectively (Bauman et al., 2006), will have an indispensible role in the steroid flux in the downstream metabolic pathways. Since 17βHSD6 catalyses the last step in the backdoor pathway to yield DHT, the activity of this enzyme towards C11-oxy C_{19} steroids is crucial to not only allow the last step in DOF in DOE metabolism in the backdoor pathway, yielding 11OHDHT and 11KDHT, but also the reactivation of the inactivated steroids within the C_{19} and C11-oxy C_{19} metabolic pathways. In the context of 21OHD, increased 11OHAST and 11KAST levels may therefore drive the steroid flux in favour of reactivation by 17βHSD6, yielding the respective active androgens 11OHDHT and 11KDHT. As discussed in chapter 5, 11OHAST is reported to be the highest C11-oxy metabolite in urinary samples of 21OHD patients, ascribed to the downstream metabolism of 11OHA4 (Kamrath, Hochberg, et al., 2012). The data presented in this study, however, clearly demonstrates that this is not necessarily the case with the metabolism of DOE and DOE via the enzymes in the backdoor pathway, certainly contributing to the 11OHAST seen and thus to the androgen pool in 21OHD.

Although the defect in the CYP21A2 enzyme, leads to an increased production of 17OHP4, DOF and A4 (Christakoudi, Cowan, Christakudis, et al., 2013), 17OHP4 is the gold standard for the identification of 21OHD. It is however apparent that this is not a reliable marker in the identification of this disease. In adults, intact gonads may contribute to androgen levels, and in females the ovaries is an additional source of 17OHP4 that can interfere with effective diagnosis and treatment (Auchus, 2015). Since 17OHP4 biosynthesis is prominent in the ovaries, levels may change significantly during the menstrual cycle. This variation is not observed with DOF, and DOF levels were markedly higher in adrenal effluents compared to the vena cava, supporting the adrenal-specific contribution of DOF (Milewicz et al., 1984). DOF has been identified as one of the best markers for 21OHD, and even though this steroid is not prominent in healthy individuals, the biosynthesis of DOF may have a central role in adrenal steroid production in 21OHD (Fiet et al., 2000; Nahoul et al., 1989). Similarly, the adrenal contribution to T production is overshadowed by the testicular production of T in males, suggesting that the A4:T ratio is a better indicator of adrenal involvement in steroid production (Auchus, 2015; Milewicz et al., 1984). It is therefore crucial to consider a spectrum of steroids to accurately diagnose 21OHD and in dispensing effective treatment. In adrenal disorders and diseases associated with the adrenal, the classical pathways in steroid production (Figure 2.3) cannot be assumed, and a more complex, integrated view is required. This was clearly illustrated in the elucidation of the 11OHA4 pathway. The finding that A4 and T produced by the adrenal contribute significantly to downstream potent androgen levels (Chang et al., 2011), together with their C11-oxy metabolites (Bloem et al., 2013; Storbeck et al., 2013; A. C. Swart & Storbeck, 2015), challenges established dogmas. The production of 11OHP4 and DOF, as well as their keto-forms, 11KP4 and DOE may contribute towards the production of potent C11-oxy androgens in a similar manner by serving as precursors to active androgens in downstream pathways, as was illustrated in chapter 5.

Taking into account the increased levels of adrenal P4 and 17OHP4 in 21OHD, together with the biosynthesis of 11OHP4 and DOF presented in this study and their conversion to 11KP4 and DOE, the C11-oxy C_{21} steroids may play a prominent role not only in precursor production in 21OHD but also as C11-oxy C_{21} hormones. Preliminary studies in our group have indicated that DOF and DOE may also activate the progesterone receptor (PR) and the AR, with DOF exhibiting partial agonist activity towards PR-B and DOE partial agonist activity towards the AR (data not shown). 11OHP4 and DOF, together with 11KP4 and DOE may therefore be unique steroid metabolites in 21OHD, serving as more specific markers for this disease. In the past, the accurate detection of these steroids was hampered due to high cross-reactivity of immunoassay based techniques (Nahoul et al., 1989). The advances in LC-MS/MS based technologies now allow the sensitive and accurate quantification of trace steroids, enabling the identification of these steroids in serum samples of 21OHD patients.

The data obtained in the cell model systems supporting the hypothesis that DOF and DOE metabolism via the enzymes in the backdoor pathway may be a prominent pathway in androgen biosynthesis, needs to firstly be fully characterised in transfected cell models, in isolation, away from competing enzymes. As

such, the conversion of 11OHPdiol and 11KPdiol by CYP17A1 requires further investigations in transfected cell models to ascertain the catalytic activity of the enzyme's lyase activity towards these substrates, currently, hampered by the lack of standards for the novel steroid metabolites. Furthermore, studies regarding the activation of C11-oxy C_{19} steroids catalysed by 17βHSD6, a prerequisite step in the backdoor pathway, needs to be fully addressed. In addition, the conjugation of the C11-oxy C_{19} and C_{21} steroid metabolites by the UGT2B enzymes also remains to be clarified. Studies in LNCaP cells have however showed that 11KT is not as readily conjugated as T or any of the other C11-oxy C_{19} metabolites (du Toit et al., 2017), with data suggesting that the C11-oxy moiety hinder effective conjugation, prolonging the cellular effects of these steroids and impacting on the interaction of these hormones with steroid receptors. The activity of DOF and DOE together with their novel metabolites towards the receptive steroid receptors are inconclusive and remain to be determined. Since DOF and DOE were demonstrated to contribute to the pool of C11-oxy C_{19} steroid metabolites, the increased production of C11-oxy metabolites in 21OHD may yield possible substrates for CYP19A1. To the best of our knowledge the aromatisation of the C11-oxy metabolites have not been reported. In the light of conditions such as PCOS associated with 21OHD and the co-expression of 17βHSD6 with estrogen receptor β (ERβ) (Muthusamy et al., 2011), the C11-oxy steroid metabolites may have a significant influence on cellular steroid profiles and cell proliferation, with the possible conversion to C11-oxy C_{18} steroids, illuminating an entire class of steroids.

It is clear that the androgen excess characteristic of 21OHD cannot be attributed to a single steroid, and the data presented in this study suggest that adrenal steroid metabolites, upon reaching target tissue, may be metabolised to a spectrum of androgens, in a scenario more complex than initially presumed, with studies only recently taking cognisance of the C11-oxy metabolites. In this study we demonstrated that the downstream metabolism of the adrenal C11-oxy C_{21} steroids gives rise to novel steroid metabolites that can contribute to potent androgen steroid levels and potentially influence 21OHD phenotypes. Advances in LC-MS/MS based technologies and the use of eloquent tools such as the UPC^2-MS/MS in screening processes in androgen dependent diseases such as 21OHD, has enabled the generation of steroid profiles which allow a more comprehensive analyses of relevant steroid hormones, rather than the analyses of single key metabolites, with the inclusion of C11-oxy steroids, is bound to have a significant impact on the diagnoses and treatment of 21OHD.

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